Quantification of gene expression in single cells

Bengtsson, Martin

2007

[Link to publication](#)

Citation for published version (APA):
Bengtsson, M. (2007). *Quantification of gene expression in single cells*. [Doctoral Thesis (compilation), Department of Clinical Sciences, Malmö]. Department of Clinical Sciences, Lund University.

Total number of authors:
1

Quantification of Gene Expression in Single Cells

Martin Bengtsson

This thesis will be defended on the 4th of May, 2007 at 13:15 in the
Medical Research Centre (Lilla Aulan), Malmö University Hospital, Malmö, Sweden.

Faculty opponent: Prof. Frans Schuit, Katholieke Universiteit Leuven, Belgium

LUND UNIVERSITY
Faculty of Medicine

Department of Clinical Sciences, Malmö

Organization LUND UNIVERSITY	Document name DOCTORAL DISSERTATION
Department of Clinical Sciences, Malmö	Date of issue 2007-05-04
	Sponsoring organization
Author(s) Martin Bengtsson	

Title and subtitle

Quantification of gene expression in single cells

Abstract

Studies of apparently homogeneous cell populations and single cells often give highly divergent results. Cells exhibit varying responsiveness to stimuli and gene expression and they are in many aspects stochastic and unpredictable. We have developed a method to measure gene expression quantitatively in individual cells with real-time RT-PCR.

mRNA for hormones, ion channels and enzymes in pancreatic alpha- and beta-cells were quantified. The distribution of transcript levels were highly skewed and was best described by a lognormal distribution. Thus, the geometric--and not the commonly used arithmetic--mean value is the appropriate measure of average expression level. In beta-cells, insulin mRNA levels were increased in response to glucose stimulation; an effect due to an increased fraction of cells with high expression. The insulin genes Ins1 and Ins2 have similar promoter regions and were indeed co regulated within single beta-cells.

Na-channels in alpha- and beta-cells display very different inactivation properties (being separated by 40 mV). We measured hormone mRNA and all Na-channel isoforms in single cells and correlated gene expression with patch-clamp recordings. Cell type-specific expression of Na-channel isoforms can partly explain the divergent inactivation.

Early differentiation of human embryonic stem cells involves the transcription factors Pou5f1, Nanog and Sox2. We quantified their expression in single stem cells and observed that they are not correlated with each other. Instead Pou5f1 correlates with the transcription factors Id1 and Id3.

We conclude that quantitative gene expression measurements on single cells allow: 1) studies of cell population heterogeneity and noise in gene expression; 2) exploration of genes that are co-regulated; and 3) correlation of gene expression with functional properties such as electrophysiological properties.

Key words:

lognormal distribution, stochastic gene expression, single cell PCR, real-time RT-PCR, Na-channels, hESC

Classification system and/or index termes (if any):

Supplementary bibliographical information:	Language English
ISSN and key title: 1652-8220; Lund University, Faculty of Medicine Doctoral Dissertation Series	ISBN 978-91-85559-41-1
Recipient's notes	Number of pages 122	Price
	Security classification	

Distribution by (name and address) Martin Bengtsson, Clinical Research Centre, UMAS Ing 72, S-205 02 Malmö, Sweden. martin.bengtsson@med.lu.se

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THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

QUANTIFICATION OF GENE EXPRESSION IN SINGLE CELLS

Martin Bengtsson

LUND
UNIVERSITY

Science may be described as the art of systematic over-simplification.

Karl Popper, 1982

Table of Contents

Original Papers

This thesis is the summary of the following studies, referred to in the text by their Roman numerals:

I   **Bengtsson M**, Rorsman P and Ståhlberg A. Single-cell mRNA quantification with real-time RT-PCR. *Submitted*

II   **Bengtsson M**, Ståhlberg A, Rorsman P and Kubista M. (2005) Gene expression profiling in single cells from the pancreatic islets of Langerhans reveals log-normal distribution of mRNA levels. *Genome Research* **15**(10): 1388-92 *(reproduced with permission from the publisher)*

III   Ståhlberg A, **Bengtsson M** and Semb H. Quantitative transcription factor analysis of undifferentiated single human embryonic stem cells. *Submitted*

IV   **Bengtsson M**, Partridge C, Remrachaya R, Salehi A, Wendt A, Zhang de Marinis Y, Braun M, Eliassson L and Rorsman P. Widely different Na^+-current inactivation properties in α- and β-cells may result from cell-specific expression of distinct Na^+-channel subunits. *Manuscript*

Abbreviations

ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
cAMP	Cyclic adenosine monophosphate
CRE	cAMP response elements
FISH	Fluorescent in situ hybridization
GLP-1	Glucagon-like peptide 1
GSP	Gene-specific priming
GTC	Guanidine thiocyanate
hESC	Human embryonic stem cells
PCR	Polymerase chain reaction
PDX-1	Pancreatic duodenal homeobox-1
qRT-PCR	Quantitative reverse transcription PCR
RT	Reverse transcription
TTX	Tetrodotoxin

Quantification of gene expression in single cells

Martin Bengtsson

Introduction

Diabetes Mellitus

The diagnosis diabetes mellitus refers to the abnormal regulation of plasma glucose. Currently, diabetes mellitus is considered to be manifest when the plasma glucose concentration exceeds 7 mM during fasting and/or 11 mM 2 hrs after a 75 g oral glucose challenge, according to the criteria set by the World Health Organization[1]. It is caused by defect insulin secretion and/or action[2]. The name *diabetes* originates to the Greek word for siphon and *mellitus* that means honey, referring to the thirst and excessive flow of (sweet) urine exhibited by untreated patients[3]. The main divider between most cases of diabetes alludes to the lack (type-1 diabetes) or presence (type-2 diabetes) of β-cells in the islets of Langerhans.

Type-1 diabetes normally presents early in life, typically following an autoimmune destruction of the β-cells, and requires replacement of insulin (insulin-dependent diabetes mellitus, IDDM). There is large geographic variation in the prevalence of type-1 diabetes, with incidence rates >20 per 100 000/year throughout most of Europe and North America, while <10 in most of South America, Africa and Asia[4]. The prevalence is increasing steadily world-wide. Scandinavia – Sweden and Finland in particular – is severely affected with incidence values as high as >30 cases/100 000 inhabitants and year. Currently, there are approximately 70 000 patients with type-1 diabetes in Sweden alone[5].

Signs of type-2 diabetes include defective insulin secretion from β-cells and typically also partial resistance to the effects of insulin in the body. Type-2 diabetes does not always require treatment with insulin

(hence its former name non-insulin-dependent diabetes mellitus, NIDDM) and is typically associated with abdominal obesity. The genetic predisposition is strong, polygenic and complex and estimated to account for 40-80% of disease risk[2]. Type-2 diabetes is classed as a pandemic and directly affects at least 300 000 people in Sweden alone and more than 150 million people world-wide. This number is estimated to reach 300 million by 2025; an increase mostly taking place in developing countries[5, 6].

On top of type-1 diabetes (representing ~15% of all cases) and type-2 diabetes (~70%), other forms of diabetes include (but are not restricted to): late-onset autoimmune diabetes in adults (LADA, ~10%)[7], direct monogenetic effects on insulin release or action (Maturity Onset Diabetes of the Young [MODY], ~5% of all DM cases)[8, 9], gestational diabetes, drug- or chemical-induced diabetes and infections causing diabetes. Genetic association and linkage analyses of the human genome lead to a growing awareness of the influence of genetic components in diabetes, and the classification of diabetes may have to adapt accordingly.

The islets of Langerhans

The pancreas is both an exocrine gland and an endocrine gland. In the exocrine part, lobules of acinar cells produce pancreatic juice that is secreted through the pancreatic duct into the small intestine, containing enzymes required for the digestion of carbohydrates, fat and protein. The endocrine pancreas is also referred to as pancreatic islets, or islets of Langerhans. They are clusters of cells dispersed among the acinar cells, each consisting of about 1000 cells. A human pancreas contains 500,000 to one million islets[10]. Their main function is to maintain glucose levels in the blood within certain boundaries, which is of high importance for the body as glucose is the main energy source for most organs and the sole supply of energy for the brain. Glucose concentration is controlled by very accurate secretion of polypeptide hormones into the blood, most notably insulin and glucagon. The islets are heterogeneous and contain phenotypically distinct cell types, such as insulin-secreting β-cells (~70% of total islet cell number), glucagon-producing α-cells (~20%) and somatostatin-releasing δ-cells (~5%). The remaining cells secrete other hormones such as ghrelin and pancreatic polypeptide. In addition, the β- and δ-cells produce islet amyloid polypeptide[11] and the α-cells synthesize peptide

YY[12]. Insulin and glucagon have opposite effects on the blood glucose level. In the body, glucagon opposes the actions of insulin and their ratio determines the intricate control of gluconeogenesis and glucogenolysis[13]. Glucagon is a catabolic hormone that triggers energy stores (mainly liver and muscle) to release glucose to the blood, while the anabolic hormone insulin stimulates glucose uptake.

Following a meal, the plasma glucose is elevated. Glucose in the blood equilibrates across the β-cell membrane via the glucose transporter 2 (GLUT2) (but note that in man accumulating data implicates GLUT1[14]). Inside the cell, glucose is phosphorylated by glucokinase forming glucose-6-phosphate that enters glycolysis and the Krebs' cycle, followed by an increase of ATP at the expense of ADP. The resultant increase in the cytoplasmic ATP:ADP-ratio closes the ATP-dependent potassium channels (K_{ATP}-channels). This causes membrane depolarization that in turn leads to opening of voltage-gated Ca^{2+}-channels. Ca^{2+}-influx via these channels and the subsequent increase in $[Ca^{2+}]_i$ at the release sites then triggers exocytosis of insulin-containing granules. This is referred to as the "triggering" pathway of insulin secretion (Figure 1). In addition, glucose exerts an amplifying effect which is exerted at a level distal to the eleva-

tion of $[Ca^{2+}]_i$. The identity of the second messenger remains to be established but there is some data implicating NADPH[15] but changes in ATP and ADP have also been proposed to be involved[16].

Insulin secretion is modulated, but not triggered, by increased cAMP levels resulting from, for example, binding of incretin hormones GLP-1 or the islet hormone glucagon (secreted by neighbouring α-cells) to receptors on the β-cell membrane. The effects of cAMP are both protein kinase A (PKA)-dependent and -independent[17] and are found to potentiate insulin secretion by stimulation of the release process itself as well as the recruitment of granules to the release sites[18]. Somatostatin inhibits insulin release through the action of hormone-specific G protein-coupled receptors culminating in the activation of the protein phosphatase calcineurin[19] and presumably dephosphorylation of key exocytosis-regulating proteins.

Much less is known about the regulation of glucagon secretion from the α-cells. However, it is clear that they are electrically excitable and that glucagon secretion associates with the generation of electrical activity. Glucagon is released by Ca^{2+}-dependent exocytosis of glucagon-containing secretory vesicles. Glucose inhibits glucagon release but the exact mechanisms involved remain

debated. For example, α-cells are equipped with K_{ATP}-channels of the same type as in β-cells. In the β-cells, closure of K_{ATP}-channels triggers membrane depolarization and insulin secretion. K_{ATP}-channels are active in the intact α-cell and their closure results in membrane depolarization[20]. This leads to the somewhat surprising conclusion that glucose leads to closure of K_{ATP}-channels in α-cells but that the resultant depolarization results in inhibition of secretion in the α-cell rather than stimulation as is the case in the β-cell. Clearly, it is essential to establish the precise ion channel complement in the α-cell.

Gene expression of insulin

The insulin gene is a small (~1400 bp) and highly conserved gene separated into three exons residing on chromosome 11 in man. The genome of mice, rats, some frogs and fish contain two actively expressed insulin genes (insulin I and insulin II) with minor base-pair differences but with identical protein sequence[21, 22]. Insulin I is likely a functional retroposon of insulin II, meaning that an RNA-mediated duplication-transposition event some 35 million years ago inserted a copy of the insulin II gene, including the promotor region, at another location in the genome. Multiple copies of genes are frequently found in the genome
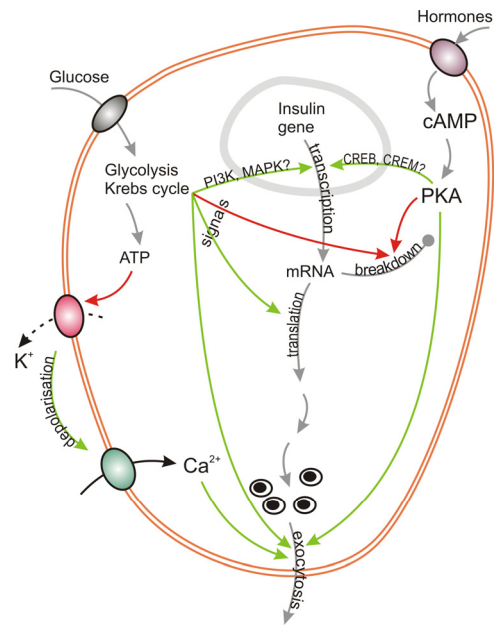
*Figure 1. Insulin release in a **β**-cell. Signals from glucose metabolism indirectly control most aspects of insulin production and release. Green and red arrows indicate a stimulating and inhibitory effect respectively. See text for full description.*

but they are normally silent, known as pseudogenes. In this case, however, both variants are abundantly expressed in the β-cell and appear regulated in unison, most likely explained by the similarities in the ~400 bp region 5' of the transcriptional start sites[21, 22]. Translation of the spliced ~600 bp mRNA generates an 11.5 kDa polypeptide called *preproinsulin* that contains four distinct parts: a signal peptide responsible for transport to endoplasmic reticulum; a peptide called chain C; and the insulin A- and B-chains. The C-peptide connects the A- and B-chain and aligns three disulphide

bridges which are essential for correct folding. Preproinsulin is cleaved to *proinsulin* by removal of the signal peptide, and it is transported to the Golgi for storage in vesicles. Inside the secretory vesicles, the C-peptide is cleaved off in a reaction catalysed by prohormone convertase and carboxy peptidase. This leaves the mature insulin protein that precipitates with zinc to form microcrystals.

Transcription of the insulin gene(s) is stimulated by glucose to replenish the intracellular stores following insulin release. In one study, β-cells were exposed to high glucose concentration for 15 minutes. Transcription of the insulin gene peaked after 30 min and cytoplasmic insulin mRNA content was 2- to 5-fold higher than basal levels 60-90 min after stimulation[23]. The fact that cytosolic mRNA levels do not mirror the transcription is a consequence of the long lifetime of the insulin mRNA molecule and that it is also under metabolic control. Other studies have observed similar effects, although the time course was slightly slower[24, 25].

Glucose metabolism alone generates signals initiating insulin gene transcription[26], possibly acting through phosphatidylinositol 3-kinase (PI3K) and mitogen activated protein kinase (MAPK)[27, 28], and is required for initiation of insulin gene transcription. The upstream region of the insulin gene contains numerous transcription factor-binding site sequence motifs. This may account for the cell-specific expression of insulin and regulation by external factors[27, 29]. Two types of motifs, termed A-boxes and cAMP response elements (CRE), are of particular importance. The A-boxes bind the pancreatic duodenal homeobox-1 (PDX-1) transcription factor, which is a major activator of not only the insulin gene, but also other islet-specific genes such as Glut2, glucokinase, IAPP and somatostatin[24]. It has been suggested that 60% of insulin transcriptional activity is dependent upon binding of PDX-1 to the A-box regulatory elements[30], and it is required for both the stimulating effect of glucose metabolism as well as the negative feedback by insulin itself[28]. PDX-1 is also considered one of the major players in early commitment of the primitive gut to pancreatic fate and in the maturation of β-cells[31].

Incretin hormones, such as glucagon-like peptide 1 (GLP-1) or glucose-dependent insulinotropic peptide (GIP), increase the intracellular concentration of cAMP by receptors coupled to adenylate cyclases[32]. cAMP regulates the transcription of genes with CREs by PKA-mediated phosphorylation of CRE binding proteins (CREB) and CRE modulators (CREM). However, the major effect of cAMP on in-

sulin transcripts is not at the transcriptional level, but is exerted by regulating mRNA stability in the cytoplasm[33]. In the presence of 17 mM glucose, the rate of degradation decreases significantly compared to 3 mM glucose[34]. Somatostatin decreases insulin mRNA stability by ~30%[35], the glucocorticoid dexamethasone also induces degradation of insulin transcripts[36]. By contrast, GLP-1 increases insulin mRNA stability in the β-cell[37, 38].

Embryonic stem cells

The human embryonic stem (ES) cells are the only cells that, to our knowledge, have the ability to transform to any human cell type (pluripotent). In addition, they can proliferate in an undifferentiated state, allowing them to renew[39]. Obviously, the potential of these cells in regenerative medicine is enormous and have been suggested as treat-

ment for a long list of diseases, one of which is diabetes[40]. However, it is a daunting feat to simulate the natural development and trigger stem cells into differentiation to primitive gut tube, pancreatic endoderm and finally a hormone secreting endocrine cell[41]. So far, these attempts have been unsuccessful but promising data were recently presented[42].

Human ES cells are derived from the inner cell mass of the mammalian blastocyst, and the transcription factors Pou5f1(Oct4), Nanog and Sox2 are key regulators the maintenance of the cells. Manipulated cells lacking at least one of these regulators and do not maintain the characteristic pluripotency and self-renewal capacity[43, 44]. Little is known about this regulation but the expression of these factors rapidly drops as differentiation is initiated.
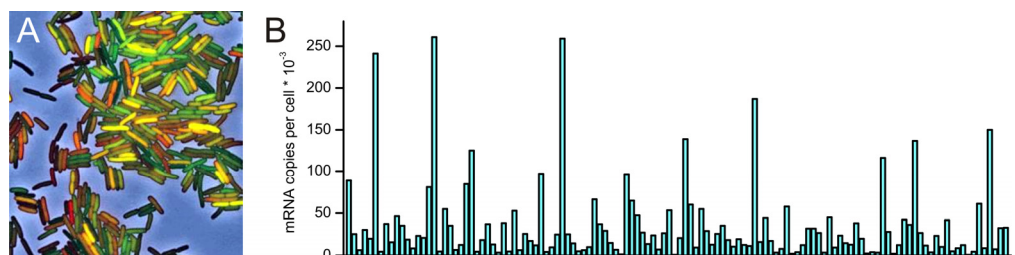
Figure 2. Cell population heterogeneity in protein (a) and mRNA (b) levels. (a) depicts E. coli expressing two fluorescent proteins (cfp shown in green and yfp in red) under the same promoters. Fully synchronous expression would give all yellow cells, but noise in the gene regulation causes variation both in the correlation and total amount of protein. Image courtesy of Elowitz et al [55]. (b) shows Ins1 mRNA levels in 125 β-cells, quantified by qRT-PCR (derived from Paper I). Ins1 is an abundant transcript and the expression levels are log-normally distributed in the population.

Single-cell biology

Cells have a remarkable ability to cooperate and jointly construct complex structures such as tissues, organs or whole organisms. These constructions are normally accurately tuned and respond to stimuli with high precision. During development, cells differentiate to specialised cell types, each with particular functions in the environment they reside. How are these actions in billions of cells coordinated? One way is for every cell to function as an exact miniature of the entire organ. The cells would act independently of each other, and respond identically to stimuli. A large bulk of data, discussed below, indicates that this is not how it works. In many aspects, individual cells exhibit a very high degree of variability and cells within a seemingly homogeneous population may exhibit great variation in the responses to identical stimuli.

Figure 2 illustrates heterogeneity in a cell population with respect to gene expression. Early indications of population heterogeneity came from studies of β-galactosidase formation in bacteria[45, 46] and human leukocytes[47, 48]. Investigations of an enhancer action using transfected reporter genes showed a 100- to 1000-fold difference in gene expression between cells exposed to identical stimuli[49]. Also in pancreatic β-cells, large cell-to-cell differences in metabolic and transcriptional responses were observed in a number of early studies[50-54] (see also Figure 3). More recently, engineered gene networks have given new insights to the cell-to-cell variation by observing expression levels of fluorescent proteins in living bacteria[55] and yeast[56, 57]. They noted two kinds of variation: 1) variable expression over time but with some different proteins varying in unison, i.e. they were correlated; and 2) uncorrelated fluctuations in expression levels (termed 'extrinsic' and 'intrinsic' noise, respectively). The word *noise* has become the collective term for all cell-to-cell variation, regardless of whether it originates from subtle environmental differences, genetic or epigenetic modifications, variation in cell states (such as cell cycle progression), stochastic fluctuations in gene transcription and (indeed) experimental variation. However, small changes in local environment is believed to have only minor effect on noise, and hereditary modifications take too long time to explain the relatively fast changes observed[58]. The idea that stochastic – random – variation has a major effect on gene expression and cell variability has been strengthened the last few years[55-57, 59-66] and today noise is often used synonymously to random fluctuations. This means that even cells with the same genome,
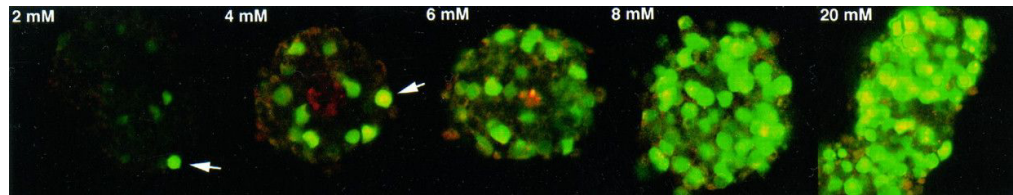
17

Figure 3. Heterogeneous gene induction. *Showed here are intact islets infected with AdIns-GFP and incubated for 48 hrs in 2–20 mM glucose. As the glucose concentration is raised, the number of GFP-positive cells increases. Picture courtesy of de Vargas et al [50].*

share the same history and that live in the same environment may display random differences – some small, other more some significant.

It was suggested already fifty years ago that when a cell is exposed to an increasing stimuli, the *probability* of a response – not the size of it – increases[46]. This is referred to as a binary, all-or-none, response, resulting in a highly heterogeneous population of cells exhibiting a bimodal distribution. Since then, this phenomenon has been reproduced several times[67-71]. Clearly, this is not the case for all mechanisms in the cell, as a graded response is seen in for example GABA$_A$ receptors[72] and Ca^{2+}-induced exocytosis in β-cells[73]. In fact, cells most likely possess the whole spectrum of responses, including graded as well as binary responses. A graded response will give rise to a gradually increasing dose-response curve, while a binary response corresponds to a sharp rise in response within a narrow dose range. In other words, sigmoidal curves with similar start- and end-points but with widely

different slope factors (n$_h$) (Figure 4). Cells may exhibit both types of regulation and cells may switch between graded and binary responses[67, 74].

Stochastic variation of gene expression

Gene expression, defined as the reactions controlling the abundance of gene products, influences most aspects of cellular behaviour. The majority of genes have only two active copies in the genome, and the initiation of transcription relies on binding of TATA binding protein, transcription factor IIB and other transcriptional activators to attract the RNA polymerase II[75]. In addition, regulatory elements mediate the degree of expression by binding to the promoter region. Prior to this, the chromatin needs to be remodelled to make the DNA accessible to the transcription machinery[76]. The origin of the stochastic variation in gene expression has not been elucidated. However, one explanation postulates that since many steps in this chain of events rely on the random en-
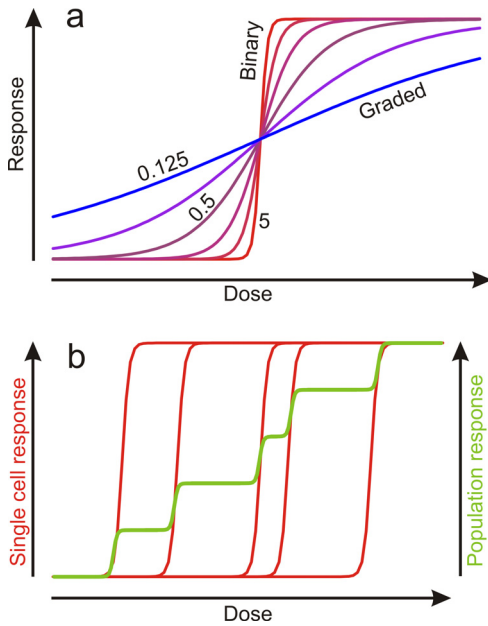
Figure 4. Sigmoidal dose–response curves illustrating graded and binary responses (a) and probabilistic response model (b). The numbers indicate slope factors (n_h). (b) shows five cells with a probabilistic binary response (red). The accumulated response for the whole population (green) approaches a graded shape as the number of cells increase. This illustrates that in large populations there is no way of telling a probabilistic from a deterministic model of gene expression.

counter of molecules, some of which are present in small quantities, the process becomes intrinsically stochastic[61, 62, 77]. There is an ongoing debate on the influence of random (intrinsic) noise on gene expression. While most studies conclude that intrinsic noise is the main contributor to the observed cell-to-cell variation, there are indications that external factors (e.g. global events affecting the whole cell to the same degree, for

example the number of polymerases, ribosomes, cell-cycle position and cell size) dominate[55, 78, 79]. Its influence could depend on the gene expression level, as low- and medium-abundance transcripts appear to be mostly affected by intrinsic noise and not so much by external factors[59, 80].

Could there be an advantage of random fluctuations in mRNA and protein levels? It probably makes cells more adjustable to rapid changes in the environment and more tolerant to stress[66, 81] – skills of central importance to a unicellular organism. Multiple steady states could be populated more quickly, allowing cell differentiation during development[82, 83]. Combined with a positive feedback loop of a particular regulatory protein, a cell population could diverge into two subpopulations by allowing random fluctuations[67, 84]. However, for some genes, random fluctuations in gene expression are likely to be disadvantageous. This includes genes required for the survival of the cell and genes that are part of important multi-protein complexes. Indeed, it has been shown that such genes have lower stochastic noise than most other genes, implying that noise is under evolutionary pressure[59, 63, 85, 86]. This indicates that the cell has a means to control noise in gene expression.

Consider a protein with low transcription rate followed by high rate of translation

and another protein with high transcription and inefficient translation. Experiments on the bacteria *B. subtilis* show that the production of the former protein generates more noise than the expression of the latter[66, 85, 86], an idea supported by theoretical models of gene expression[62, 87]. Similarly, experiments in yeast suggest that more efficient promoter activation would decrease noise levels[56, 57, 88]. The inclusion of a negative feedback loop (such as a transcription factor affecting its own expression negatively) can also be envisaged to reduce noise[89, 90]. Another way for cells to lower noise is to increase the number of gene copies in the genome[66], suggesting a possible explanation to why mice and rats have two functional insulin genes – it decreases noise. Noise reduction is probably energetically costly for the cell and switching between noisy states and less variable conditions may be a way for cells to minimize the unfavourable effects of noise while still making use of its benefits and save energy[91].

Two recent studies provide the largest sampling of single cell gene expression to date[59, 63]. The researchers investigated protein noise properties in yeast and found that the noise of a particular gene is inversely proportional to the mean expression level of that gene, indicating that fluctuating mRNA levels is the major source of protein noise

while gene activation and mRNA translation do not vary considerably between cells. This suggests that mRNA numbers are generally low (often 1-2 molecules, [63]), resulting in a Poisson distribution of transcripts, and the rate of translation high (~1200 proteins produced per mRNA, [59]). In one of only a few studies on mammalian cells, Raj *et al* show a somewhat different picture in higher eukaryotes than what is observed in bacteria and yeast [65]. They measured mRNA molecules with high precision, showing evidence of 'transcriptional bursting', i.e. short periods of massive mRNA production, supporting the idea that *gene activation* is the major source of noise. Similar conclusions are drawn from experiments showing transcriptional events in living cells, in real-time[83, 92-94]. A likely explanation for transcriptional bursts is chromatin remodelling, implying that it is not the mRNA production per se that is responsible for the observed noise, but upstream events. However, bursting is observed also in bacteria (that lack chromatin structure) so there must be other factors involved[93]. Raj *et al* also observes that up-regulation of a gene generates larger bursts, but with unaltered frequency, somewhat contradicting the assumption that stronger stimulation increases the likelihood of expression initiation[64, 68, 70]. Burst-like behaviour of gene expression of genes

required for cell survival might appear hazardous, but since the half-lives of proteins are generally much longer than that of mRNA molecules, the fluctuations on mRNA level are buffered.

In conclusion, single-cell noise is a phenomenon that, although predicted early[95], has not been possible to investigate in detail until recently. The underlying mechanisms are only starting to come forth, but it is clear that stochastic variation is a fundamental property of cell physiology in general and gene expression in particular. Emerging technologies allowing quantitative, non-interfering measurements of single molecules in (ideally) living cells will help "clear the fog".

Aims of the study

The purpose of this work was primarily to establish a method for detecting and quantifying mRNA transcripts in single cells and to combine this method with patch-clamp recordings on the same cells. The specific goals were to:

1. correlate electrophysiological characteristics to gene expression profiles (hormones, ion channels, etc) in pancreatic islet cells;

2. elucidate the discrepant Na^+-current inactivation properties in α- and β-cells by single-cell measurements of Na^+-channel isoforms;

3. study transcriptional noise in pancreatic islet cells and tumour cell line, and determine how widely cells in a population differ from each other;

4. investigate the effect of increasing glucose concentration on insulin and glucagon mRNA levels in single cells and confirm or rule out bimodality; and

5. characterize early differentiation of human embryonic stem cells by studying key transcription factors and their correlation within individual cells.

Methods

Much of the work behind this thesis lies in the development of the methods used. The choice of the appropriate technology, detection chemistry, reagents and conditions requires thorough testing and optimization. Specific detection of minute amounts of mRNA is currently only possible with two methods: Fluorescent In-Situ Hybridization (FISH) and the Polymerase Chain Reaction (PCR), and variants thereof. They both rely on single stranded oligonucleotides base-pairing with the intended target, but from there they go separate paths. In FISH, labelled probes allow localization of mRNA in cells fixed on microscope slides and coupled with a signal amplification strategy, such as Tyramide Signal Amplification (TSA)[96] or tandem array probe binding sites[97], detection of single mRNAs can be achieved[65, 98]. Traditionally, the PCR runs in a solution and does not allow collection of spatial or temporal data, as the cell is normally sacrificed during analysis (with some exceptions [99]). Generally, PCR detects its target more specifically than FISH due to the single sequence-recognizing element (the probe) used in FISH while PCR uses two or three (primers and probe). In addition, the temperature cycles of PCR greatly increases specificity compared to iso-thermal hybridization. Variants of FISH to assay mRNA in living cells (Fluorescent In Vivo Hybridization, FIVH) have been presented using molecular beacon probes[100], RNA aptamers [101], and MS2 tandem repeats together with the fusion protein MS2-GFP[83, 93, 101-103]. A concern with all experimental methods in living cells is that the hybridization of probes may interfere with the translation or degradation of mRNA.

Initially, we tested both FISH- and PCR-based methods for single cell mRNA assessment, but soon settled for the PCR-method due to its superior sensitivity, specificity, compatibility with patch-clamp measurements, and higher quality of quantitative data. We developed methods to collect single cells and reliably isolate RNA that ensured high success-rate and good yield. Figure 5 shows an overview of the procedures and below are all steps described in detail:

Patch-clamp measurements

The patch-clamp technique is the most versatile method to measure ion fluxes, membrane potential, and changes in membrane capacitance in single cells. Currents (in the pA- to nA-range) are recorded by electrodes positioned on either side of the cell membrane and amplified by ultra-sensitive electronic amplifiers. Measurements are of two
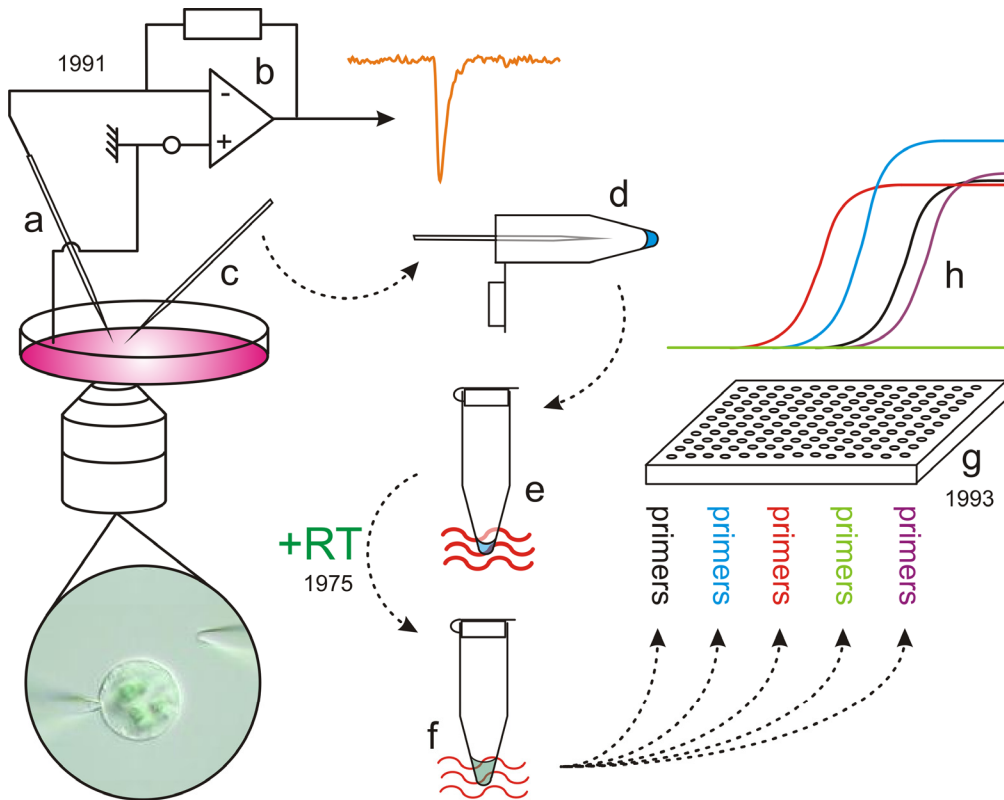
Figure 5. Patch-clamp recording and expression analysis on the same cell. *A patch pipette (a) is used to record the membrane currents from a single cell that are amplified in the patch-clamp amplifier (b). The electrode is positioned on the cell surface using a micromanipulator (the process viewed via the objective of the microscope). Using a dedicated cell collection pipette (c), the intact cell is brought to a test tube (d) containing 2 µl lysis buffer. The tube is incubated at 80 °C for five minutes (e) and allowed to cool. At this stage the sample may be stored at -80 °C for extended periods of time. A reaction mixture containing the reverse transcriptase enzyme, dNTP, buffer, RNase inhibitor and RT-primers is added to the sample and incubated at 37-55 °C (f), according to the chosen temperature protocol. The resulting cDNA is split into vessels containing PCR-primers and PCR mastermix, which subsequently are temperature cycled (g) with simultaneous fluorescence detection giving readout in the form of amplification curves (h).*

Numbers indicate the years a Nobel Prize was rewarded: In 1975 Howard Temin and David Baltimore received the Prize in Physiology or Medicine, partly for their discovery of the reverse transcription enzyme; in 1991 Erwin Neher and Bert Sakmann were given the Prize in Physiology or Medicine for the development of the patch-clamp technique; and in 1993 Kary Mullis was presented with the Prize in Chemistry for the invention of the polymerase chain reaction

kinds: 1) current-clamp, in which the membrane potential is recorded; and 2) voltage-clamp, where the current crossing the membrane is recorded. A glass capillary shaped as a pipette with a tip diameter in the µm range, mounted on a micromanipulator, allows connection with the inside of the cell. In this thesis the standard whole-cell configuration was used for all measurements (Paper IV). In this recording mode, the membrane patch between the pipette and the cell is ruptured thereby permitting pipette contents to enter the cell and *vice versa*.

Single-cell collection and lysis

Compared with cell culture and patch-clamp recording, RT-PCR and other methods of molecular biology have different needs and standards when it comes to cleanliness and purity. While infection is a great threat for the cells in culture, it is normally a minor issue for molecular work on nucleic acids. On the other hand, factors unimportant for cell work, such as nucleases (e.g. DNases and RNases that are very abundant in the pancreas) and enzymatic inhibitors are a source of "headache" in molecular biology. The cell itself also contains enzymes capable of degrading mRNA, and there are numerous proteins binding to nucleic acids making them less accessible for the primers, probes and enzymes used by the researcher. Most

importantly, the mRNA itself can have strong secondary structures obstructing the analysis[104]. In addition, we were confronted with the problem of getting the cell from the culture dish into an RT-PCR compatible test tube.

Some reports indicate that it is possible to collect mRNA from cells by sucking the cytoplasm into the patch-clamp pipette[105-108]. We abandoned the idea early due to low yield and poor reproducibility with pancreatic islet cells, although there are cases where it remains the sole option (i.e. when recording from intact pancreatic islets) which works albeit with a low success rate. Instead, we used a dedicated cell-collection borosilicate glass pipette to collect the intact cell. This has the advantage that the cellular mRNA is exposed to degradation for shorter time and that the complete cell, and not just a part of it, is collected. The pipette was emptied in an RNase- and DNase-free tube using a custom-made device, basically a micromanipulator holding the pipette and a solid tube holder mounted on a support.

The cell is emptied into a lysis buffer. We evaluated a number of methods to disrupt the cell membrane and make the mRNA accessible for reverse transcription, some of which are presented in Paper I. Our experience was that no single method, such as repeated freeze-thaw cycles, proteinase K

treatment, detergents or heat incubation, is essential for successful analysis, but some – most notably detergents and heat incubation – improve yield. We also evaluated the presence of carriers, inert molecules that limit adverse adhesion effects by covering surfaces, without noting any big effect. In the end, we recommend low concentrations of the strong protein denaturant guanidine thiocyanate combined with brief heat incubation as the preferred choice for cell lysis and mRNA preparation. Standard laboratory practice suggests purification of the mRNA prior the reverse transcription[109]. However, after evaluation of this step we feel that the risk of losing sample was higher than the potential gain, especially as we saw no inhibitory effects of down-stream reactions of either the lysis buffer, extracellular buffer or the cell itself.

Reverse transcription

The enzymatic reaction of converting RNA to DNA is referred to as "reverse transcription" (RT), and is catalyzed by RNA-dependent DNA polymerases known as reverse transcriptases. These enzymes are naturally occurring in retro-viruses, and most enzyme species available on the market today originate from the Moloney Murine Leukemia Virus (MMLV) and Avian Myeloblastosis Virus (AMV)[104]. MMLV-based

RT-enzyme has a natural RNase H activity, potentially causing sample degradation[110], which has promoted the development of genetically engineered enzymes lacking this feature[111]. The action of reverse transcriptases is similar to that of other DNA polymerases. The reverse transcription is initiated with the binding of a single stranded oligonucleotide to the mRNA, serving as a starting template for the reverse transcriptase. The transcriptase elongates the oligonucleotide, commonly referred to as 'RT-primer', in the 5' to 3' direction by adding free nucleotides (dNTP) complementing the mRNA sequence and creating a DNA:RNA hybrid helix. Thus, the DNA sequence (cDNA) is the reverse complement sequence to the mRNA and it can be used as template in PCR (see below).

The efficiency of the RT-reaction is highly variable and unpredictable, depending on choice of enzyme, RT-primer, temperature protocol and reagent concentrations[112-115]. However, as shown in Paper I the reaction is highly reproducible if the conditions do not change between experiments, emphasizing the need of identical reaction conditions throughout a study[116]. The nature of the target mRNA influence the reaction yield greatly, in particular the secondary structure[104, 117]. A high incubation temperature reduces secondary struc-

tures, which has motivated many enzyme manufacturers to engineer RT-enzymes that are more tolerant to heat, allowing incubation temperatures up to 55 ℃. Attempts to reduce mRNA secondary structures are presented and discussed in Paper I. It should be pointed out that the absolute quantification in Papers I-III is based on the assumption that the efficiency of the reverse transcription is constant and close to 100%. RT-efficiency is measured with RNA standards and our previous study indicates efficiencies up to 80% with the reaction conditions used[115]. This means that our estimates of mRNA copy number will be slightly lower than the true value.

Analysis of mRNA from individual cells put the reverse transcription reaction to test. Accurate quantification requires high and consistent yields. To detect signals from rare transcripts (sometimes down to single mRNA molecules), the enzyme must reverse transcribe with good accuracy. Template accessibility and removal of inhibitors were discussed in the previous section, but also in the pure sample the potential of optimization of the RT reaction is great. We assessed the effect of RT-priming (both its kind and concentration) and temperature profile (Paper I). RT-primers are available as stretches of thymine nucleotides (oligo(dT)), random sequences or oligonucleotides targeting spe-

cific genes (gene-specific primer, GSP). Oligo(dT) primers, usually 15-18 nucleotides long, chiefly bind to the poly-adenine tail attached to 3'-end of most mRNAs. In theory, the products generated from RT with oligo(dT) primers are a complete stretch of cDNA containing a single copy of the entire gene. In reality however, this is not the case due to mRNA degradation and unspecific binding of primer. Still, oligo(dT) is considered the first choice of RT-primer[118]. Random primers, usually six nucleotides long and referred to as random hexamers, bind randomly throughout the transcriptome and the ribosomal RNA. This strategy is suggested for poor quality RNA, but the risk of generating multiple cDNA copies of the mRNA has given rise to concerns about the quality of the quantitative data[119]. However, our data are reassuring in this context and indicate that random hexamer priming does not generally give higher reaction yield than for example oligo(dT) (Paper I). Gene-specific priming ideally results in a homogeneous cDNA of only the intended target. The disadvantage is the cDNA can only be used for a single target, limiting its use for rare samples where analysis of multiple genes is required.

Nucleic acid hybridization is the basis for binding of both RT- and PCR-primers, but the process is far from exact and predict-
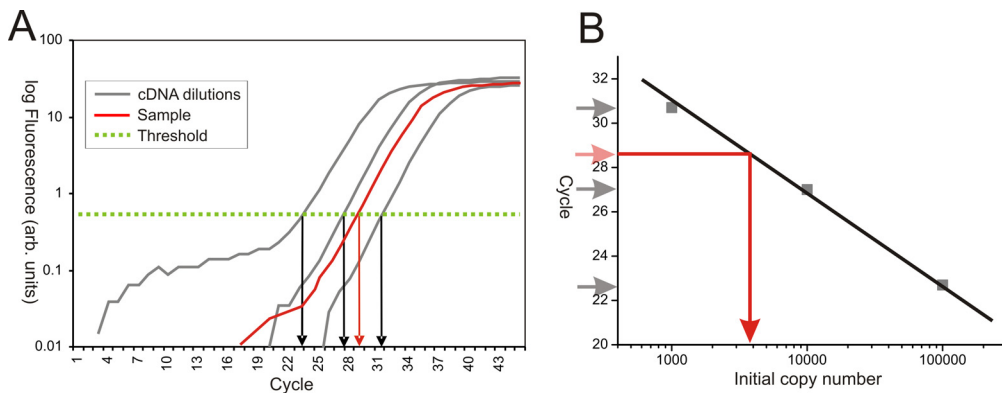
Figure 6. Quantification of DNA templates with PCR. (a) shows the exponential fluorescence increase (logarithmic scale) due to PCR amplification of three samples with known starting copy number (grey) and one unknown sample (red). A fluorescence threshold is set arbitrarily and the number of PCR cycles it takes for the amplification curve to reach this threshold is termed the cycle of threshold, Ct (arrows). A standard curve (b) is derived by connecting the Ct-values of the known samples plotted against the initial copy number. The unknown sample is quantified by reading the copy number corresponding to the Ct-value (red arrow).

able adding another degree of complexity to the RT-PCR process. Complementary oligonucleotides join or separate in a stochastic manner. External factors, such as temperature and ionic strength, affect the probability of a particular hybridization. The melting-point of a particular sequence is perhaps a misleading description as it only denotes the temperature at which 50% of the strands are hybridized (which is the same as saying that the strands are base-paired 50% of the time). Non-complementary base-pairing is seen in for example micro-RNA[120], demonstrating the flexibility of nucleic acid hybridization. This effect is particularly pronounced in reverse transcription where the reaction temperatures are low. It is therefore not surprising that products that should *not* form

using a particular priming strategy nevertheless are found and often at fairly high levels (Ståhlberg A, personal communication). Even reverse transcription without RT-primers cDNA generates products. This probably occurs by using the mRNA itself as primer and illustrates the random nature of the reaction.

Quantitative PCR

Real-time PCR, or quantitative PCR (qPCR), was derived from the classical polymerase chain reaction in the early 1990s[121] by simultaneous monitoring of the DNA template amplification process. The temperature cycling, heat-stable Taq polymerase enzyme and oligonucleotide primers resulting in an exponential increase

of templates are all identical in traditional PCR and qPCR, but the latter is complemented with a passive probe allowing the progress of the reaction to be monitored in "real-time". Thus, the benefit of PCR (the detection of rare DNA sequences in a complex mixture) is preserved while the time-consuming post-processing of samples by gel electrophoresis is avoided. The quantification is based on the linear relationship between the fluorescence of bound probe and the template concentration. At the start of the qPCR, the fluorescence is below the detection limit, but as the number of templates increase exponentially, so does the fluorescence. Depending on the amount of starting material, the number of PCR cycles it will take to reach an arbitrarily set threshold (termed cycle of threshold, Ct) will vary (Figure 6)[112, 122, 123]. Quantification using qPCR is intrinsically *relative* to other samples, it does not provide an absolute copy number in a single experiment. Absolute values are estimated by comparing the Ct-value of an unknown sample with that of a sample with known amount of starting material, in practice by the use of a standard curve[122, 124].

The commonest form of quantification of gene expression levels with qRT-PCR makes use of internal reference genes, sometimes called house-keeping genes. By relating the expression level to a gene assumed to be constant in the samples being analyzed, the expression of the gene of interest can be determined in terms of the ratio between the two genes. This strategy makes the choice of internal reference gene a crucial one, potentially affecting the conclusion drawn from the experiment. Incorrect normalization has turned out to be one of the most frequent mistakes in qPCR analysis[116, 118, 125, 126] and motivated the use of more than one gene for reference[127] or normalization to ribosomal RNA[128] or total RNA amount[118].

Quantification as described above is based on a number of assumptions, most notably that of constant PCR efficiency. In theory, the PCR doubles the number of template copies in each round of amplification. In reality this is usually slightly less than a doubling in each cycle, varying between genes and reaction conditions. An efficiency close to 100% is desired but it is even more important that the efficiency is *constant* in every experiment with the same primers, or else the quantification will be inaccurate. In fact, it is the same assumption also for the reverse transcription reaction efficiency, emphasizing the importance of identical reaction conditions throughout a study.

The probes for template detection are categorized into two groups: 1) fluorescently labelled oligonucleotide probes binding specifically to a predetermined sequence; and 2) fluorescent dyes recognizing any amplified DNA product. The most frequently used sequence recognizing probe is the hydrolysis probe, known as TaqMan®[129], based on a design with two excitable molecules; a donor dye and a quencher connected by an oligonucleotide. When in close proximity, the emission from the donor dye is absorbed by the quencher by energy transfer. During the amplification reaction, the Taq polymerase cleaves the probe, thereby separating the dyes allowing detection of the donor dye emission wavelength. Ideally, this results in a 1:1 relationship between the number of templates and free donor dye. Variations on this theme include the hair-pin shaped Molecular Beacon[130] and the single-dye LightUp probe[131]. A recent review[123] discusses this aspect in depth.

The other group of reporters used in qPCR are dyes that recognize any double-stranded DNA, and increase or shift their fluorescence upon binding. Asymmetric cyanine dyes, such as the popular SYBR Green I[132] and BEBO[133], bind to the minor groove of the DNA helix, resulting in increased fluorescence intensity. The drawback is that they recognize any DNA, not just the

template targeted by the oligonucleotide primers, but also unwanted products such as primer dimers resulting from incomplete base-pairing between primers. Elimination of primer dimers are important regardless the choice of detection chemistry as they in high concentrations interfere with the intended amplification reaction[134]. However, unless the fluorescence is read at temperatures above the melting point of the primer dimer[135, 136], the primer dimers will generate an amplification curve only when using reporter dyes.

Conveniently, the reporter dyes allow in-tube analysis of the amplified product after the PCR has completed, providing
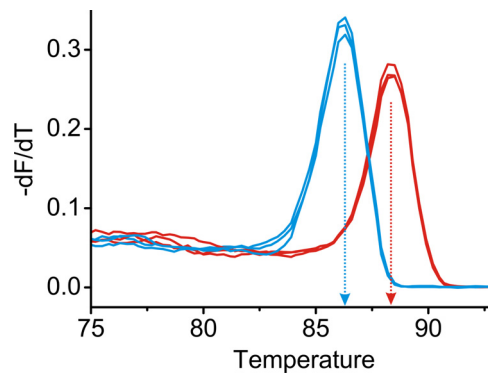
Figure 7. Melting curves of two different PCR-products, using SYBR Green I as reporter dye during a gradual increase in temperature. As the temperature reaches the melting point of the product (T_m), the helical structure collapses, resulting in a sharp drop in fluorescence as the dye is separated from the DNA. Shown here is the negative derivative of the fluorescence decay. Consequently, the curve peaks at the T_m of the PCR-product (indicated by arrows).

similar information as agarose gel electrophoresis. By collecting fluorescence at temperatures near the melting point of the amplified products, a melting curve (also referred to as dissociation curve) is generated (Figure 7). When the helix collapses at the melting temperature of the product, a sharp drop in fluorescence is observed, allowing distinction of products based on melting point[137]. This distinction can be very accurate and used to separate products with only small differences in size or sequence[138], and primer dimers are generally easily discriminated from the desired product.

When the starting material is limited, such as in single-cell PCR, the number of genes that can be accurately measured is small, especially for low-abundance genes. Parallel amplification reactions in a single tube, referred to as multiplex PCR amplification, followed by standard qPCR, leads to a vast improvement in this respect. The multiplex reaction requires special considerations[139] and generally uses much lower primer concentrations than the 2nd reaction, used for detection and quantification. In Paper IV, we use a variant coined 'preamplification', allowing up to 100 assays to be run in parallel with starting material from a single cell.

In this summary the focus is on the methodology of the single-cell qRT-PCR measurements. Experimental details pertaining to the other methods used (immunocytochemistry, cell culture, hormone release measurements, etc.) are given in the individual papers (I-IV).

Results

Paper I

Single-cell collection and lysis

Paper I contains a detailed description of a protocol for quantification of mRNA in single cells with qRT-PCR. It represents an effort to define the optimum conditions, reagents and concentrations in each of the steps to quantify mRNA from individual mammalian cells. These protocols were applied to dissociated pancreatic islet cells, but they are by no means limited to this tissue. The cell collection method is, however, not applicable to work on intact tissues. Borosilicate glass pipettes, traditionally used for patch-clamp measurements, were fabricated with tip diameters matching cell size. Detergents and proteinase K treatment for cell lysis were evaluated by adding a single islet and measure the amount of mRNA liberated. It was concluded that proteinase K had little effect and guanidine thiocyanate (GTC) provided efficient disruption of the islet and cell membranes. Next, we tested the

compatibility of the detergents with the downstream reverse transcription reaction. As expected, high concentrations (>100 mM) of GTC severely inhibited the reaction while low concentrations (~40 mM) surprisingly resulted in a 3- to 6-fold increased reaction yield compared to control conditions. This finding may be of interest for all applications of reverse transcription, not just single-cell qRT-PCR.

Optimizing the reverse transcription

An effort was made to ensure optimal conditions for reverse transcription. We evaluated priming with random hexamers, oligo(dT), gene-specific primers, and combinations of them. In addition, three temperature protocols were tested: isothermal, gradient and cycled incubation temperature. Although the variation between identical experiments was small, yields varied up to 5-fold between different priming strategies. However, it was difficult to draw any general conclusions from these results as there was no trend common for all tested genes. This is in agreement with previous results[114], showing that RT-priming is highly gene-specific and individual optimization is required for maximum yield. In most experiments with gene-specific primers, unspecific products were generated. This illustrates the importance of not using gene-specific primers
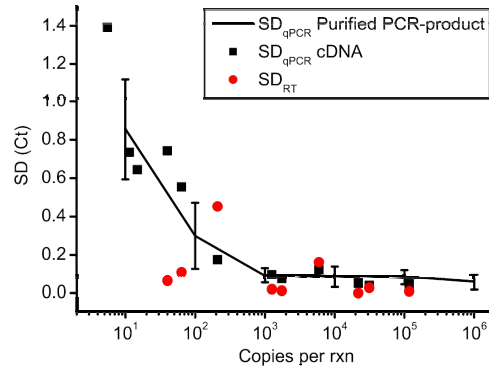
Figure 8. Reproducibility of RT and qPCR. The standard deviations of triplicate PCR- and RT-reactions (squares and circles) are low for starting amount above ~100 copies. At lower concentrations the spread increases drastically, in part due to Poisson effects but also due to the intrinsic inaccuracy of the exponential amplification in PCR. The reproducibility is not improved when using purified PCR-product as starting template (solid line) instead of cDNA (squares).

without first carefully testing the specificity of the reaction. We conclude that a combination of random hexamers and oligo(dT), both at 2 μM, will work well for all but the most difficult templates and it was used throughout the rest of the study.

Technical reproducibility

For a quantitative method to be useful it has to demonstrate high reproducibility in the range for its intended use. Figure 8 shows standard deviations, originating from RT and qPCR respectively, at varying template concentrations. At starting amounts above ~100 copies, the spread is low, allowing accurate quantification. Below 100 copies, the

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SD increases sharply and repeated measurements of a sample containing 20 copies would span around 10-40 copies. This is too inaccurate for most purposes. We emphasize that this is an effect inherent to the PCR method itself and it is not due to the treatment or an artefact from the reverse transcription, since dilution of purified PCR product generated data with similar spread (Figure 8). Furthermore, standard deviations of replicate measurements on single cells also ended up in the same region. We conclude that at mRNA copy numbers less than ~100 (assuming a measurement of ~20% of the total single cell cDNA[116]), this method should be used primarily for detection and not for quantification purposes.

Single-cell measurements on glucose stimulated α- and β-cells

We applied the method to single α- and β-cells with 96% success rate and measured gene expression of insulin I (*Ins1*), insulin II (*Ins2*), glucagon (*Gcg*), ribosomal protein S29 (*Rps29*) and chromogranin B (*Chgb*) in each cell. Expression levels were highly heterogeneous and skewed towards higher numbers. They all fit the lognormal distribution (discussed in Paper II), thus making the geometric mean the most appropriate measure of average values. Correlation coefficients between all genes confirmed that the transcription of *Ins1* and *Ins2* correlate,

which is expected as their promoter regions are identical. The cells were exposed to increasing concentrations of glucose resulting in elevated expression of the insulin genes. High glucose exerted its effect by affecting a small fraction of cells with insulin expression >10-fold above average. For example, of the cells containing the highest *Ins1* (top 20%) transcript levels, 90% had been incubated in high glucose (10 and 20 mM). Still, a majority of the cells were apparently not affected by glucose. The expression of *Chgb* was close to the limit of accurate quantification, yet we observed stimulation by glucose at the single-cell level as well as on population level. In summary, we demonstrate a method that makes possible the isolation of mRNA from single mammalian cells and that generates reliable quantitative data for expression levels down to ~20 transcripts per reaction.

Paper II

Lognormal distribution of transcript levels

Paper II describes the distribution of transcripts in pancreatic β-cells and correlates expression levels of five genes within single cells. The main finding is that mRNA levels for a particular gene are lognormally distributed within a population. We measured both pancreatic β-cells and MIN6 mouse insulinoma cells and they all had highly skewed

histograms of expression levels, resembling a lognormal distribution. The distributions were positively identified as lognormal with statistical tests for lognormality. Thus, the geometric mean is the appropriate method to calculate the typical expression level of a cell (see Discussion below). In addition, we report that the expression of *Ins1* and *Ins2* are correlated in single cells, in agreement with the finding in Paper I. As expected, we observed a strong induction of both insulin genes in high glucose (20 mM) compared to low concentration (5 mM). Surprisingly, β-actin was also up-regulated by glucose. β-actin is a commonly used reference gene used for normalization of gene expression data, but this result questions its suitability as reference in β-cells.

Paper III

Transcription factors in human embryonic stem cells

In Paper III, we measured the expression of key transcription factors in single human embryonic stem cells (hESCs). These cells have the ability to differentiate into any human cell type and form the basis for future replacement therapies to treat diseases such as diabetes and Alzheimer's disease. However, the signals directing the differentiation are not known in full. This study is an attempt to shed light on the initial stages of differentiation. Six genes were measured: the transcription factors *POU5F1*, *NANOG* and *SOX2*, known to regulate pluripotency and self-renewal in hESCs and that are not expressed in differentiated cells; and the inhibitor of DNA binding-genes (named *ID1*, *ID2* and *ID3*) that are important in mice but of unknown importance in human cells.

Similarly to what was seen in pancreatic islet cells and insulinoma cells, we observed large variations between individual cells both at mRNA and protein level. We found that the expression of *POU5F1*, *NANOG* and *SOX2* was uncorrelated in single cells, indicative of separate regulatory pathways. Instead, the expression of *POU5F1* correlated with that of *ID1* and *ID3*. The transcript distribution in the population was not lognormal for all genes; an effect of the unusually low expression levels of some cells in the population, possibly because some cells had embarked on the road of differentiation. The method used thus offers means to distinguish differentiating cells from undifferentiated at a very early stage.

Paper IV

Na⁺-channels in pancreatic α- and β-cells

In Paper IV, patch-clamp recordings were combined with single-cell qRT-PCR, focusing on the possible differential expression of
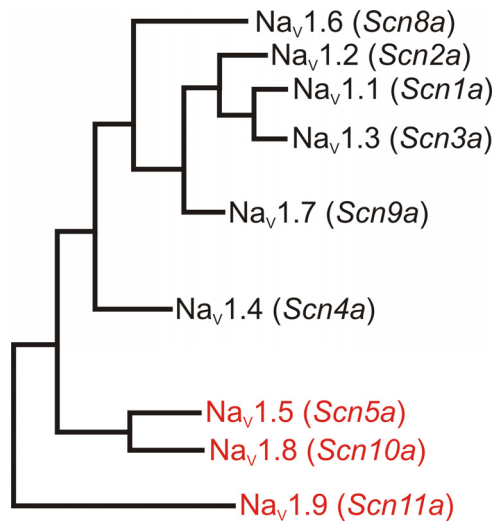
Figure 9. Phylogenetic tree of Na⁺-channel α-subunits. *Gene names are shown in parenthesis and TTX-resistant channels in red.*

voltage-gated Na⁺-channels in α- and β-cells. First, we isolated RNA from intact islets and measured all isoforms of the voltage-gated Na⁺-channels (Figure 9), both the nine isoforms of the pore-forming α-subunit and the three auxiliary β-subunit isoforms. In many excitable endocrine cells, Na⁺-channels underlie the membrane depolarization that is required for activation of voltage-gated Ca²⁺-channels that in turn trigger Ca²⁺-dependent hormone release[140, 141]. We have previously shown that the Na⁺-channels in the different islet cell types have widely varying inactivation properties[142, 143], a fact commonly used to determine cell-type during patch-clamp recording. The purpose of this study was to shed light on this discrepancy of the Na⁺-channels in the islets of Langerhans and to test whether it reflects cell-specific expression of distinct Na⁺-channel subunits.

We confirmed our previous findings of early inactivation of Na⁺-channels in β-cells by patch-clamp recordings in concert with hormone expression measurements using both immunocytochemistry and single cell PCR. Half-maximal inactivation ($V_{0.5}$) was approximately -100 mV for β-cells and -60 mV for α-cells. Whole islets and mouse insulinoma cells (MIN6) were screened for Na⁺-channel isoform expression. The α-subunit *Scn9a*, and to some degree also *Scn3a* and *Scn8a*, were present in islets and MIN6 cells, as well as the β-subunits *Scn1b* and *Scn3b*. In agreement with these data and confirming that both α- and β-cells express tetrodotoxin (TTX)-sensitive Na⁺-channels, TTX completely blocked the Na⁺-currents in both α- and β-cells. However, insulin secretion was unaffected by TTX while it reduced the glucagon release by ~60% at low glucose concentrations. The failure of TTX to affect insulin secretion from mouse islets is in accordance with earlier reports[144] and is probably a consequence of the Na⁺-channels being fully inactivated at physiological membrane potentials.

Cell material from individual cells only provides enough material for a limited num-

ber of genes, depending on transcript abundance. To allow measurement of all twelve channel isoforms in a single cell – plus the hormones insulin, glucagon and somatostatin – we utilized a pre-amplification strategy, as described in the Methods section. The expression pattern was not obvious and most (~70%) analyzed cell lacked detectable levels of Na$^+$-channel transcripts. However, data indicate that of the α-subunits, *Scn9a* dominates in β-cells and is present in some α-cells as well, while *Scn3a* is almost α-cell-specific. Roughly 80% of the β-cells with detectable levels of β-subunits expressed *Scn1b* and only ~20% Scn3b. In the α-cells, this relationship was reversed with *Scn3b* being the dominant β-subunit.

Discussion

Variable mRNA levels

Our measurements confirm the high cell-to-cell variability observed with other methods. What are the underlying mechanisms behind the noisy expression pattern observed in individual α- and β-cells and what are the implications? Possible explanations include (but are not restricted to): 1) transcriptional bursting, i.e. infrequent and fluctuating promoter activity resulting in pulses of mRNA production; 2) variable transcription production unrelated to promoter activity, with Poisson distributed mRNA levels; 3) constant mRNA production but variable degradation; or 4) sub-populations of cells with varying transcriptional capacity. These possibilities are considered in turn below.

1) Transcriptional bursting

Quantitative RT-PCR provides snap-shots of the mRNA expression profile in single cells and do not reveal the rate at which transcript levels change over time. Thus, we can not directly elucidate whether transcriptional bursting takes place in islet cells or not. The underlying mechanism behind transcriptional bursting is believed to be the binary nature of the gene promoter state: it is either *on* or *off*. Consider a promoter of a particular gene that is randomly turned on for short periods in time, while being off most of the time, based on stochastic interactions between DNA and promoter complexes or chromatin remodelling. The resulting bursts of mRNA production will be short and unsynchronized among cells in a population. Only a few cells will have an abundance of mRNA at any single point in time, whilst most cells will only have transcripts from past bursts that are being degraded. Since the mRNA degradation is likely to be concentration-dependent, the resulting distribution of transcripts per cell

will be highly skewed and resemble the log-normal distributions that we observe in our data (Paper I-III).

In β-cells, the reported half-life of insulin mRNA is ~29 hrs in low glucose and ~77 hrs in high glucose conditions[34]. This means that burst frequency would have to be lower than the reported frequencies (minutes to hours, see below) to explain the widely different expression levels. If the bursts are frequent and transcript degradation inefficient, then cell-to-cell variation would be smaller than what is observed.

Do our data on insulin gene induction by glucose fit the transcriptional bursting model? As mentioned in the introduction, the evidence is conflicting whether the burst frequency or the burst size (or both) increases when stimulated. If burst size alone was affected, the highest expression levels of stimulated cells would exceed that of the highest levels of non-stimulated cells. Conversely, if burst frequency was increased, the highest expression levels would be the similar for low and high glucose, albeit the number of cells with high expression levels would have increased. Our data, in particular Paper II, tend to support the latter hypothesis. We found that high glucose increased the number of cells with the high mRNA-levels whereas the actual amounts in these high-level cells were similar regardless of the glu-

cose concentration.

Bimodal distributions of mRNA levels [67, 84] could also be explained by transcriptional bursting. Constant burst size and longer periods of promoter activation will give distributions approaching a bimodal distribution. This applies especially if the promoter is 'leaky'. This leakiness would mean that the gene is transcribed to a low degree even when the promoter is in its inactive state. However, we do not see any evidence of bimodality of insulin transcript levels in β-cells.

2) The Poisson model

How many genes are expressed in a single cell at a single point in time, and to what degree? This fundamental question has only been addressed occasionally and it has been suggested that most transcripts are present at a very low level, on average only 1-2 mRNA molecules or less per cell[63, 145-150]. In an
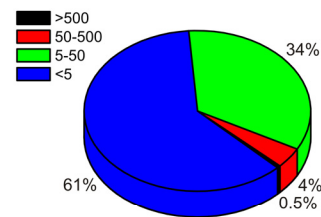

Figure 10. Transcript abundance in pancreatic tumour cells. Most transcripts are present at low copy numbers, and only a small fraction have >500 copies per cell. Data derived from Zhang et al[150].

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analysis of colon cancer cells, only 61 genes were expressed at more than 500 mRNA copies per cell[151] and in another study 75% of the genes were present at less than one copy per cell[145] (see also Figure 10). Assuming random fluctuations in the production and degradation of transcripts, the distribution will fit the Poisson distribution. This hypothesis[152] was recently shown to be valid in *Saccharomyces cerevisiae*[59, 63], for all but the most abundant genes. It was also found that this variation is the major source of noise in gene expression, and Bar-Even *et al* present a number describing the constant rate of translation (1200 proteins per mRNA[59]). At first glance, these results are not in concordance with our data, at least not in Papers I-III. However, the expression levels in our studies are much higher, and the conclusions on mRNA levels are indirect observations based on protein measurements. The Poisson model may be appropriate for low-abundance genes, but for medium expression and above it fails to explain the great variation observed. In our studies, the hormones fall into a special class. Insulin is by far the most abundantly expressed gene in the β-cell[153] with tens of thousands of copies per cell[33, 154] (Paper I-II), and the distribution is clearly not Poisson.

3) Variable mRNA degradation

Consider that the promoter of a particular gene is in its active state at all times and that mRNA is being produced at a more or less constant rate. Highly variable mRNA degradation could then generate snap-shots similar to the ones we observe in our data. Degradation could vary as a result from fluctuating levels of ribonucleases or other compounds affecting mRNA stability[155]. This aspect has not been considered much to date, and studies on single cells have not been presented.

Degradation of insulin mRNA in β-cells has been shown to be regulated by glucose[33]. There can therefore be little doubt that variations in degradation contributes to the heterogeneity. For rare transcripts, variable mRNA degradation probably contributes as much to the total noise as variable mRNA production. Nevertheless, it is unlikely that degradation is the sole source of noise for abundantly expressed genes.

4) Differentiated sub-populations

Another possible explanation to the heterogeneity we see in our data is that some cells are predisposed to produce more mRNA than others. Such a variation would be characterized as a form of extrinsic noise, although they may reflect permanent differences in cell behaviour originating from the

development of the tissue. Possibly, a cell could shift states, determined by factors such as age and localization in the tissue, or it may occur by random. It has been reported that β-cells maintain insulin production for extended periods of time[156-158]. In fact, β-cells could be divided into glucose-responsive and glucose-unresponsive groups, based on the effect of glucose treatment on insulin secretion. However, the total hormone content did not vary, only the ability to release insulin[54]. In a heterogeneous population of β-cells, each triggering more or less binary at different glucose concentrations, an increase in glucose would correspond to a recruitment of insulin-producing cells[50, 53]. Measurements of transcriptional bursting in living amoeba cells indicate that reinitiation of expression was more likely than *de novo* initiation, implying the presence of a 'transcriptional memory'[83]. In summary, there is convincing evidence that there are prolonged differences in protein level and cellular activity between cells. These variations should not be classified as noise, but as characteristics different sub-populations. However, it is difficult to ascertain that these variations are permanent and rule out slow fluctuations, as cells would need to be monitored for a very long time. The frequency of oscillations that have been reported derive from studies on dividing cells

and range from minutes[83, 94] to several hours[92, 159].

Our data cannot distinguish temporary from permanent variation in mRNA levels. We did not see any effect on the spread of transcript levels of the incubation time after islet cell preparation, indicating that if heterogeneity stems from sub-populations it is not strongly affected by cell dispersion. In addition, the variation of mRNA levels in insulinoma cells and human embryonic stem cells (Paper II-III) is not easily explained by divergent sub-populations, as they have no physiological milieu that may cause these differences.

Implications of variable mRNA levels

It is at the functional protein level that gene expression has its effect and mRNA, albeit being a messenger molecule, is by itself of no practical use to the cell. Although the correlation between mRNA levels and corresponding protein levels has been debated many times[160, 161], the assumption that high mRNA levels correspond to high protein levels is generally correct at the population level. It is a different story in single cells. It is unlikely that the fluctuating mRNA amounts in cells directly relate to the number of active proteins. The main reasons are twofold: 1) mRNA generally has a much

shorter half-life than its corresponding protein[48, 155, 162] and; 2) each mRNA generates more than one protein molecule[59, 63, 163]. Numerous attempts have been made to create theoretical models for this process[61, 78, 86, 164], and although some have proven useful in specific cases, they are not yet generally applicable.

Alternative methods

Our results show the applicability of quantitative RT-PCR to accurately measure mRNA down to ~20 copies per reaction. At lower levels the spread in the measurements increases drastically, to a point where the data become garbled. The technical reproducibility is very good at levels >100 copies with only minor variations. Large dynamic range is a distinguishing mark for qPCR; virtually no other method can unbiased quantify 100 copies in one sample and 10^9 copies in the next. However, for accurate quantification of low-abundance mRNA, other methods are more appropriate. Warren *et al* present parallel picolitre-PCRs of single cells with end-point measurements, spotting a simple yes-or-no answer from the wells that contained a single template[165]. They thus avoid the need of efficient PCR amplification and show that the method is reliable for quantification of 1-500 copies. Baner *et al* used the rolling-circle amplification of

padlock probes, an isothermal replication reaction using polymerase that generates long repetitive sequences of the probe that can be detected *in situ* or *in solution*[166]. While these alternative methods substitute the signal amplification with another technique, they still rely on reverse transcription to cDNA by reverse transcriptase.

The expression of Na$^+$-channels in the α- and β-cells (Paper IV) is very low, maybe less than one copy per cell on average meaning that not all cells contain transcripts even though they clearly possess functional channels (data not shown). At this level, quantification is not reliable and it is difficult to distinguish the technical failures from the cells that lacked transcripts. However, due to the high specificity of the method (here we have three sequence-recognizing elements), we are confident that the cells that did give rise to a signal did in fact contain the indicated Na$^+$-channel isoform.

Lognormal distributions of mRNA levels

Random distributions are often assumed to be normally distributed without being thoroughly investigated. The normal, or Gaussian, distribution arises when stochastic errors accumulate by addition while lognormal distributions on the hand set in when errors are multiplied[167-169]. Lognormal distri-
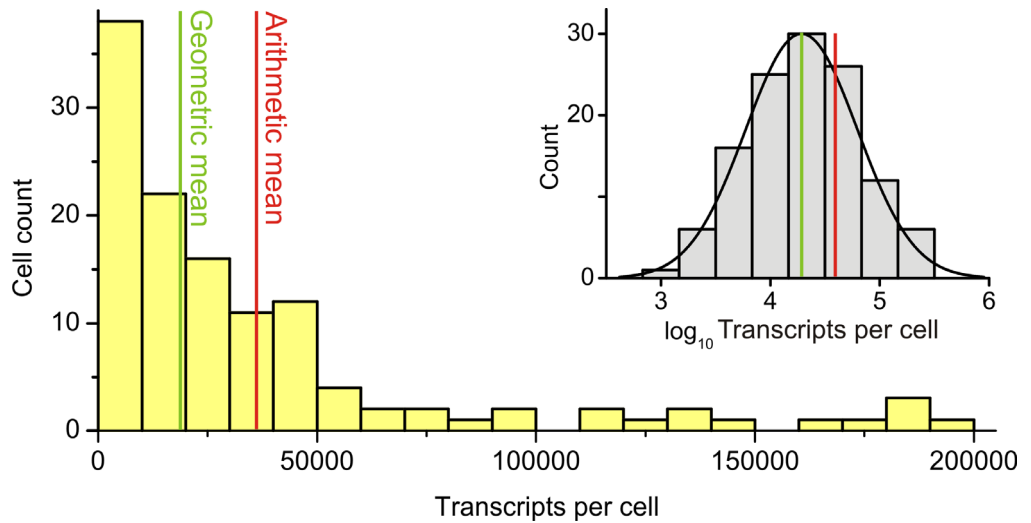
Figure 11. Lognormal distribution of transcript levels in a population of cells. Histograms of Ins1 expression level shown in linear and logarithmic (insert) scale. Green lines indicate the geometric mean, while red lines represent the arithmetic mean value. The arithmetic mean is heavily influenced by the few cells with very high expression level.

butions are frequent in nature[169, 170] and are characterized by its long tail towards higher values giving a few samples a disproportionately large influence on a measurement on the whole population. Thus, the arithmetic mean, $[(\Sigma\ X_n)/N]$, is an inappropriate estimate of the typical cell in a population exhibiting a lognormal distribution. Instead, the geometric mean, $[(\Pi\ X_n)^{1/N}]$, should be used[169] (Figure 11). Bulk measurements on whole populations of cells correspond to the arithmetic mean and single-cell measurements are required to calculate the geometric mean. The difference between arithmetic and geometric means can be large, as demonstrated by the effect of glucose on β-cells (Paper II). The fold-increase

of *Ins1* going from 5 to 20 mM glucose was 4.6 when using arithmetic mean values (corresponding to bulk measurements) while it was 17 when comparing geometric means. The latter value is a better estimate of the true induction, as the former is influenced by the few but highly expressing cells in low glucose[171].

Other reports of lognormal distribution of mRNA levels[65, 165, 170, 172-174] have followed and it appears to be frequently occurring in various cell types. It is worth pointing out that the spread originating from the exponential amplification is also lognormally distributed, just like the biological variations we observe. However, the biological spread is several magnitudes larger than

the PCR-induced variations. In addition, similar distributions are seen on data collected with non-PCR-based methods and the single-cell correlations we observe support the validity of our results.

Correlated gene expression

The correlation factors (here we used Pearson (Paper I-II) or Spearman (Paper III), the latter being independent of distribution) indicate that some genes are synchronously expressed (Figure 12). The correlation factors can tell us something about the contribution of noise from intrinsic and extrinsic sources. High correlation factors between all genes would indicate that global extrinsic noise is dominating as these factors would affect all genes simultaneously. In contrast, our data generally exhibit poor correlation between genes, indicating low extrinsic noise. However, we probably see an example of promoter- or pathway-specific extrinsic noise as the *Ins1* and *Ins2* genes correlate (Paper I-II). The relatively low variation between *Ins1* and *Ins2* is intrinsic noise, while the remaining variation between the other genes cannot be categorized into pathway-specific extrinsic noise or intrinsic noise from our measurement. Our data does not support finding that genes located on different chromosomes do not correlate[65, 80], since in mouse the *Ins1* gene is located

Figure 12. Correlation of transcript abundance in 45 single β-cells. Expression of five genes (β-actin, insulin I, insulin II, Sur1, and Kir6.2) are shown with different shades of grey. The scale is normalized so that the highest expression of each gene is black and the lowest is white. The Pearson correlation coefficients for these genes are shown in Table 2 in Paper II.

on chromosome 19 and *Ins2* on chromosome 7. Instead, factors exerting their influence prior to transcription initiation of the *Ins1* and *Ins2* are likely to be the main contributors of noise in insulin gene expression. Such factors may include the levels of transcription factors and/or unknown signals derived from glucose metabolism.

Perspectives

Recent technological progress has enabled insight into fundamental biological processes, but it is clear that much more research is needed to fully elucidate the gene regulatory systems in cells. This has become one of the focus areas of a rapidly growing field of research; systems biology, that aims to explain biology with complex models spanning all areas of physiology and molecular biology. While there are many suggested models of gene expression, more data—preferably with temporal resolution—is needed to fully confirm or dismiss the suggested mechanisms behind for example transcriptional bursting or lognormal distributions.

There are sporadic whole-transcriptome studies on single cells using microarrays[175, 176]. The data presented in this thesis warrant care when interpreting such data. Does a snap-shot of the global gene expression really provide any biologically meaningful information? Since there is no typical "average" cell[177], conclusions must be drawn from an average of a large sample set. Besides, the global amplification methods available have yet to prove they are sufficiently reliable for *quantitative* single cell analysis. However, if these obstacles are overcome, the method can provide spectacular data on how genes are regulated.

Single-cell data from all steps in the central dogma—mRNA, protein and function—could provide hints of how cells calibrate delicate cellular mechanisms, in particular if these entities could be monitored in a bigger context, like a cell population, tissue or organism. New methods, and combinations of such (e.g. patch-clamp recordings combined with multiplex imaging techniques), might assist in the quest to understand how the tiny, intricate and delicate machines in our body—the cells—really work.

Conclusions

We have developed a method for quantifying mRNA in single cells and successfully applied it on pancreatic islet cells, insulinoma cells and human embryonic stem cells. All steps were carefully optimized to reach highest possible reaction yield and accuracy. Our results give insight into basic characteristics of gene transcription and how cells in a population jointly respond to stimuli. We conclude that deterministic simulations/explanations do not give the whole picture; heterogeneity and stochasticity should be accounted for in studies of cellular mechanisms.

The following conclusions were reached:

1. Gene expression measurements can be correlated with patch-clamp recordings in individual α- and β-cells, providing solid support for (for example) cell type specific expression of hormone mRNA;

2. Na^+-currents in α- and β-cells showed discrepant inactivation properties, which in part may be explained by differential expression of distinct Na^+-channel isoforms;

3. mRNA transcription levels of a wide array of abundant-to-intermediately expressed genes are lognormally distributed in islet cells and the MIN6 insulinoma cell line. The population is thus highly heterogeneous and a small proportion of the cells contain the bulk of the total amount of transcripts;

4. Regulation of hormone mRNA levels by glucose does not appear to be bimodal. Glucose increases the fraction of cells with very high transcript levels while the majority of cells are seemingly unaffected; and

5. Transcript levels are highly heterogeneous also in human embryonic stem cells. Early signs of differentiation were observed as a drastic drop in expression of the transcription factors *POU5F1*, *NANOG* and *SOX2*, although their expression is not co regulated in undifferentiated cells.

Populärvetenskaplig sammanfattning

Allt levande som går att se med blotta ögat består av flera celler, en samling mer eller mindre oberoende enheter. En av biologins största frågor är hur dessa celler kan samspela och koordinera sig själva för att bilda komplexa organismer eller organ. Ett sådant organ är bukspottskörteln (pankreas) som innehåller hormonfrisättande klasar av celler som kallas de *Langerhanska cellöarna*. Deras funktion är främst att upprätthålla blodsockernivån inom hälsosamma gränser. Är blodsockret för lågt finns det risk för koma och hjärnskador; är det för högt skadas bl.a. kärl, nerver och njurar. Nivån styrs av β-cellerna i de Langerhanska cellöarna genom intrikat utsöndring av hormoner, i synnerhet *insulin*. Insulin verkar i kroppen för att öka upptaget av socker ur blodet, vilket leder till sänkt blodsockerhalt. Misslyckas β-cellerna med att kontrollera blodsockret får man *diabetes*, en sjukdom som nästan en halv miljon svenskar lider av. De övergripande målen med vår forskning är att förstå hur β-cellen och andra celler i de Langerhanska cellöarna fungerar, samt varför diabetes uppstår och hur sjukdomen kan förebyggas eller botas.

Vi har utvecklat en metod för att kunna studera detta i större detalj än tidigare: En cell åt gången. Denna upplösning ger oss möjligheter att även se på β-cellens roll i gruppen, om alla celler bidrar lika mycket till insulinproduktionen eller om det skiljer sig åt. I denna avhandling presenteras resultat som visar att β-celler reagerar mycket olika på samma blodsockerhalt. Aktiviteten i insulingenen kan skilja sig tusenfalt mellan två celler i samma population. Faktum är att oavsett vilken gen och vilken cell vi studerade, så såg vi väldigt stora skillnader. Endast en bråkdel av cellerna stod för majoriteten av produktionen vid en given tidpunkt. En trolig förklaring är att genuttryck är en slumpartad process och att cellerna omväxlande slås på och av.

Genom att kombinera två metoder för att studera enskilda celler kan vi få ytterligare information om sambandet mellan genuttryck och cellens funktion. Vi mätte flödet av natrium in i cellen, genom en jonkanal. Natriumkanalens genuttryck kunde därefter mätas och korreleras till dess aktivitet. Detta gav oss viktig information om hur bl.a. β-cellen fungerar som enhet, och inte bara som grupp. Återigen visar sig skillnaderna vara markanta mellan hur populationen beter sig och hur cellerna reagerar en och en. Detta belyser vikten av att studera enskilda celler och denna avhandling presenterar en metod som gör det möjligt.

Acknowledgements

All this work required help, input, inspiration and support from many people. In particular, I wish to thank:

Patrik Rorsman, my witty supervisor, for inviting me to Lund (it turned out to be a rather long stay) and giving me full scientific freedom; it was courageous and honourable of you and very instructive for me. Your enthusiasm is contagious!

Mikael Kubista, for ideas and inspiration at the early stage of the project. Thanks for all support and for introducing me to research!

Anders "Pelle" Ståhlberg, the volcano of new ideas—some brilliant, some crazy—for being a great mate in and outside of the lab. I truly enjoy working with you. The end result is never what one expected, and I mean that in a good way.

Present and former labmates in Malmö and Lund: **Anders L, Anders R, Anna, Catta, Dai-Qing, Dina, Helen, Jalal, Javier, Jenny, Jovita, Juris, Lotta, Mark, Omid, Rosita, Sandra, Steffi, Sven, Vikas, Xing-Jun** & **Yang**. For all the laughs over the years, you make Mondays worth looking forward to!

Erik, Lena, Bryndis and **Albert** for great company and inspiration, and for pulling the strings in the lab. You have served as much appreciated mentors for me.

Britt-Marie & **Kristina,** for *actually* pulling the strings in the lab, and for keeping up a cordial atmosphere.

The **Oxford-group, Matthias** and **Quan** in particular, for great science and great fun.

The people at **TATAA Biocenter**.

Anders Malmström, Birgit Liss, Henrik Semb & **Hindrik Mulder**.

Maud & **Göran**, for all the love, encouragement and freedom.

Sofia & **Henning,** ♥ $^\infty$

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