



UPPSALA
UNIVERSITET

*Digital Comprehensive Summaries of Uppsala Dissertations
from the Faculty of Medicine 1189*

Single Molecule Detection

Microfluidic Automation and Digital Quantification

MALTE KÜHNEMUND



ACTA
UNIVERSITATIS
UPSALIENSIS
UPPSALA
2016

ISSN 1651-6206
ISBN 978-91-554-9498-8
urn:nbn:se:uu:diva-279372

Dissertation presented at Uppsala University to be publicly examined in B41, BMC, Husargatan 3, Uppsala, Friday, 22 April 2016 at 14:00 for the degree of Doctor of Philosophy (Faculty of Medicine). The examination will be conducted in English. Faculty examiner: Professor Sten Linnarsson (Karolinska institute, Department of Medical Biochemistry and Biophysics).

Abstract

Kühnemund, M. 2016. Single Molecule Detection. Microfluidic Automation and Digital Quantification. *Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine* 1189. 57 pp. Uppsala: Acta Universitatis Upsaliensis. ISBN 978-91-554-9498-8.

Much of recent progress in medical research and diagnostics has been enabled through the advances in molecular analysis technologies, which now permit the detection and analysis of single molecules with high sensitivity and specificity. Assay sensitivity is fundamentally limited by the efficiency of the detection method used for read-out. Inefficient detection systems are usually compensated for by molecular amplification at the cost of elevated assay complexity.

This thesis presents microfluidic automation and digital quantification of targeted nucleic acid detection methods based on padlock and selector probes and rolling circle amplification (RCA). In paper I, the highly sensitive, yet complex circle-to-circle amplification assay was automated on a digital microfluidic chip. In paper II, a new RCA product (RCP) sensing principle was developed based on resistive pulse sensing that allows label free digital RCP quantification. In paper III, a microfluidic chip for spatial RCP enrichment was developed, which enables the detection of RCPs with an unprecedented efficiency and allows for deeper analysis of enriched RCPs through next generation sequencing chemistry. In paper IV, a smart phone was converted into a multiplex fluorescent imaging device that enables imaging and quantification of RCPs on slides as well as within cells and tissues. KRAS point mutations were detected (i) *in situ*, directly in tumor tissue, and (ii) by targeted sequencing of extracted tumor DNA, imaged with the smart phone RCP imager. This thesis describes the building blocks required for the development of highly sensitive low-cost RCA-based nucleic acid analysis devices for utilization in research and diagnostics.

Keywords: single molecule, digital, rolling circle amplification, magnetic particle, padlock probe, microfluidics, resistive pulse sensing, lab on chip, mobile phone microscopy, enrichment, sequencing

Malte Kühnemund, , Department of Immunology, Genetics and Pathology, Rudbecklaboratoriet, Uppsala University, SE-751 85 Uppsala, Sweden.

© Malte Kühnemund 2016

ISSN 1651-6206

ISBN 978-91-554-9498-8

urn:nbn:se:uu:diva-279372 (<http://urn.kb.se/resolve?urn=urn:nbn:se:uu:diva-279372>)

*Not everything that counts can be counted,
and not everything that can be counted counts.*

Albert Einstein

To my family and friends

List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

- I **Kühnemund, M.**, Witters, D., Nilsson, M., Lammertyn, J. (2014) Circle-to-circle amplification on a digital microfluidic chip for amplified single molecule detection. *Lab on a chip* **14**, 2983-2992
- II **Kühnemund, M.**, Nilsson, M. (2015) Digital quantification of rolling circle amplified single DNA molecules in a resistive pulse sensing nanopore. *Biosensors & bioelectronics* **67**, 11-17
- III **Kühnemund, M.**, Sharif, I., Hernandez-Neuta, I., Cornaglia, M., Gjeis, M., Nilsson, M. Microfluidic enrichment and targeted sequencing of rolling circle amplified single molecules. *Manuscript*
- IV **Kühnemund, M.***, Wei, Q.*, Darai, E., Wang, Y., Hernandez-Neuta, I., Tseng, D., Ahlford, A., Ozcan, A., Nilsson, M. *In situ* detection of KRAS point mutations and targeted DNA sequencing with a mobile phone. *Manuscript*. **Equal contribution*

Reprints were made with permission from the respective publishers.

Related work by the author

- I Clausson, C. M.*, Arngarden, L.*, Ishaq, O., Klaesson, A., **Kühnemund, M.**, Grannas, K., Koos, B., Qian, X., Ranefall, P., Krzywkowski, T., Brismar, H., Nilsson, M., Wählby, C., Söderberg, O. (2015) Compaction of rolling circle amplification products increases signal integrity and signal-to-noise ratio. *Scientific reports* **5**, 12317. **Equal contribution*
- II Mezger, A. *, **Kühnemund, M.***, Nilsson, M., Herthnek, D. (2015) Highly specific DNA detection employing ligation on suspension bead array readout. *New biotechnology* **32**, 504-10. **Equal contribution*
- III Saharil, F. Ahlford, A., **Kühnemund, M.**, Skolimowski, M., Conde, A., Dufva, M., Nilsson, M., Brivio, M., van der Wijngaart, W., Haraldsson, T. (2013) Ligation-based mutation detection and RCA in surface un-modified OSTE+ polymer microfluidic chambers. *Transducers & Eurosensors* **27**, 357 – 360
- IV Zelano, J., Mikulovic, S., Patra, K., **Kühnemund, M.**, Larhammar, M., Emilsson, L., Leao, R. N., Kullander, K. (2013) The synaptic protein encoded by the gene Slc10A4 suppresses epileptiform activity and regulates sensitivity to cholinergic chemoconvulsants. *Experimental neurology* **239**, 73-81

Contents

Introduction.....	11
Analysis of biomolecules	11
Analysis of nucleic acids.....	12
Sequence analysis of nucleic acids	12
Targeted nucleic acid analysis	13
Probes for ligation- and polymerization-based detection	15
Single molecule quantification methods	17
Hybridization-based single molecule quantification	17
Enzymatic single molecule quantification.....	18
Rolling circle amplification	20
Circle-to-circle amplification.....	22
Magnetic particles in RCA assays.....	23
Assay automation	25
Microfluidics	25
Continuous-flow microfluidics.....	26
Digital microfluidics.....	27
Single molecule read-out.....	28
Digital RCA read out	28
Resistive pulse sensing	29
Present Investigations	31
Paper I. Circle-to-circle amplification on a digital microfluidic chip for amplified single molecule detection	31
Background.....	31
Summary.....	31
Discussion.....	32
Paper II. Digital quantification of rolling circle amplified single DNA molecules in a resistive pulse sensing nanopore	33
Background.....	33
Summary.....	33
Discussion.....	34
Paper III. Microfluidic enrichment and targeted sequencing of rolling circle amplified single molecules	35
Background.....	35
Summary.....	35
Discussion.....	36

Paper IV. <i>In situ</i> detection of KRAS point mutations and targeted	
DNA sequencing with a mobile phone.....	37
Background.....	37
Summary.....	37
Discussion.....	38
Perspectives	39
RCA read-out methods – a comparison	39
Conclusions and outlook	42
The future of C2CA.....	42
The future of RCA read-out.....	42
Acknowledgements.....	43
References.....	48

Abbreviations

A	Adenine
ASM	Amplified single molecule
ASMD	Amplified single molecule detection
bDNA	branched DNA
bp	Base pair
C	Cytosine
C2CA	Circle-to-circle amplification
cDNA	complementary DNA
CV	Coefficient of variance
DMF	Digital microfluidics
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
ELISA	Enzyme-linked immunosorbent assay
EWOD	Electrowetting-on-dielectric
FISH	Fluorescent <i>in situ</i> hybridization
G	Guanine
hRCA	Hyperbranched RCA
HRP	Horse radish peroxidase
LOC	Lab-on-a-chip
LOD	Limit of detection
MDA	Multiple displacement amplification
MIP	Molecular inversion probe
μTAS	Micro total analysis system
NA	Nucleic acid
NGS	Next generation sequencing
OLA	Oligonucleotide ligation assay
PCR	Polymerase chain reaction
PDMS	poly(di-methyl siloxane)
POC	Point-of-care
qPCR	Quantitative polymerase chain reaction
RCA	Rolling circle amplification
RCP	Rolling circle amplification product
RNA	Ribonucleic acid
ROI	Region of interest
RPS	Resistive pulse sensing
SBH	Sequencing by hybridization

SBL	Sequencing by ligation
SBS	Sequencing by synthesis
smFISH	single molecule fluorescent <i>in situ</i> hybridization
SNP	Single nucleotide polymorphism
T	Thymine
T _m	Melting temperature
U	Uracil

Introduction

Much of recent progress in medical research and diagnostics has been enabled through advanced molecular analysis technologies, which allow biomolecule detection and quantification with high specificity and sensitivity. Basic biomedical research has expanded our understanding of the molecular etiology of disease, a knowledge that is increasingly becoming the fundament for modern diagnostics and personalized medicine. The development of new diagnostic methods is essential to translate this knowledge into improved diagnoses and selection of therapy.

Our laboratory has recently developed biomolecule detection assays that provide a high level of specificity and enable the most accurate mode of quantification possible: single molecule quantification. However, so far, the sensitivity of these assays has been fundamentally limited by the efficiency of the detection method that is used for read-out. Additional amplification steps can compensate for inefficient detection systems, but usually add complexity to an assay. The rapidly expanding field of microfluidics increasingly facilitates medical research and cost-effective diagnostics through the reduction of reaction volumes and assay automation in miniaturized formats.

This thesis describes microfluidic approaches that enable automation and digital quantification of nucleic acid detection assays based on rolling circle amplification (RCA). I will give an overview of related molecular assays with a focus on digital assays, and continue with microfluidic principles and sensing technologies, with focus on those that are relevant for my own work. In the second part, papers that constitute this thesis will be reviewed and discussed. In the final section, I will review recent developments on RCA-based detection methods in comparison to my own technological contributions.

Analysis of biomolecules

In order to understand the components of life, we need to study the diversity, abundance, modifications and interactions of biomolecules. Carefully studying changes in any of these parameters can give important information about the health status of a being. Proteins and nucleic acids are the most widely studied biomolecules due to their fundamental roles in building, maintaining

and changing a living organism. Proteins are the building blocks of life, whereas nucleic acids (NA) are the carriers of information about how to build life, although, as we recently start to understand, NAs also have essential regulatory functions (1). Certain proteins and nucleic acids, and their alterations, have proved to be especially useful to monitor health and diagnose disease. They are called biomarkers. In this thesis, I will focus on nucleic acids and present improved strategies for their usage as biomarkers.

Analysis of nucleic acids

Analyzing nucleic acids is attractive due to their relatively simple molecular structure, composing only four building blocks, the nucleotides, comprising a sugar backbone, a phosphate group and one of four nucleobases Adenine, Guanine, Cytosine and Thymine or Uracil (A, G, C and T (U instead of T in RNA)). The sequence in which these building blocks are connected, encode certain functions. There are numerous methods available for the detection of particular nucleic acids and for the analysis of nucleic acid sequences.

Sequence analysis of nucleic acids

In the process of nucleic acid sequencing, the exact base composition of RNA or DNA sequences can be determined through a variety of sequencing methods. The first methods, that allowed longer sequence reads of nucleic acids, were published nearly simultaneously by Sanger (2) and Gilbert (3) in 1977, both awarded with Nobel prizes in 1980. Although Gilbert's chemical sequencing method initially gained more popularity, Sanger's method, based on chain termination during polymerization of the complementary strand, was simpler to automate. The same methodology was used in the first automated DNA sequencing machines from ABI and delivered the first human genome sequence in 2001 (4, 5). Sequencing by synthesis (SBS) chemistry is still used today in next-generation sequencing (NGS) instruments. The advancing steps from the first to the second generation of sequencing technologies are (i) the reversion of the chain termination which allows continuing sequencing on the same strand, and (ii) the isolation and clonal amplification of individual DNA molecules and the subsequent massively parallel sequencing of clonally amplified single molecules.

This resulted in a tremendous increase in throughput and decrease of costs (6-9). NGS started in 2005 when the company 454 Life Sciences demonstrated genome-sequencing in micro-wells (10), using pyrosequencing chemistry (11). Several other technologies for NGS have been proposed since then, including sequencing by hybridization (SBH) (12, 13) and sequencing

by ligation (SBL) (14). Both SBH and SBL chemistries were later adapted to sequence rolling circle amplification products (15, 16).

Now we are at the verge to the 3rd generation of sequencing technologies entering the market. These methods perform single-molecule sequencing without clonal pre-amplification. Examples are nanopore sequencing (17-19), now commercialized by Oxford nanopores, and single-molecule real-time sequencing (SMRT) (20), commercialized by Helicos and Pacific Biosciences. In contrast to the 2nd generation sequencing technologies, which deliver short 50-200 bp sequence reads and heavily rely on complex sequence alignment algorithms, single molecule sequencing allows very long read-lengths facilitating *de novo* sequencing. The upcoming 4th generation of sequencing applies 2nd generation sequencing technology directly in cells and preserved tissues, offering NA sequence analysis with spatial resolution (21, 22).

Targeted nucleic acid analysis

With many whole human genomes sequenced, and many genetically caused conditioned diseases identified, we now have a large genomic inventory that allows distinguishing health from disease. Moreover, pathogenic organisms, and their potential resistances to drugs, can be identified based on particular sequences within their genetic material. This knowledge allows defining particular sequences of interest and designing molecular assays that focus on exactly those NA molecules and regions within NA sequences that are relevant to a certain application. Reconstruction of the entire genetic information of an organism is, in most cases, not required, and only generates an unnecessary amount of data and costs.

Targeted NA analysis is more rapid, cost-effective and enables a more thorough analysis of a particular sequence of interest. In targeted sequencing, particular regions of interest are isolated, enriched and sequenced with higher sequence coverage and increased read depth, i.e. many NA molecules of the same kind are sequenced. This enables detection of rarely occurring sequence aberrations that would otherwise be missed in whole-genome sequencing (with less read depth). Other targeted analytical approaches simplify the level of obtained information by circumventing reading sequences, and instead delivering only quantitative information about a particular NA molecule and, if required, detecting sequence aberrations. I will briefly outline the most commonly used targeted NA detection principles and go in more depth when describing digital quantitation formats.

Hybridization

Based on the basic principle of Watson-Crick base-pairing specific NA sequences can be detected by hybridization of sequence complementary hy-

bridization probes, which can be modified with reporter molecules, such as radioactive isotopes, enzymes and fluorophores. Some of the still used pioneering techniques are southern and northern blot for DNA and RNA detection, respectively (23) (24). Samples and cell extracts are transferred and immobilized on a membrane, detected by hybridization with sequence-specific probes and visualized by a reporter molecule. In the late 1980s, DNA microarrays were developed that enable the quantitative assessment of nucleic acid content in a sample through massively parallel hybridization on pre-manufactured arrays with printed DNA probes (25-27). The main difference as compared to blotting is that the sample nucleic acids themselves are labeled before hybridization to the array. Nucleic acids can also be visualized *in situ*, i.e. directly inside cells and tissues, through *in situ* hybridization (28, 29), and later through fluorescent *in situ* hybridization (FISH) (30).

Ligation

In 1988, a ligation-based approach was developed which made use of the fidelity of T4 ligase to discriminate single nucleotide substitutions by oligonucleotide ligation assay (OLA) (31). This principle was crucial for the development of sequencing by ligation chemistry. In ligation amplification reaction (LAR) and ligation chain reaction (LCR) this reaction is cycled with intermediate denaturation leading to exponential increase of the ligation product (32, 33). In 1994, OLA was extended by connecting the two separate interrogation probes through a linker sequence. These linear, so-called pad-lock probes (34), are described in detail later.

Polymerization

The power of polymerization-based approaches is that minimal amounts of NAs can be detected specifically and with relatively high speed through amplification. Today's gold standard in polymerization-based NA detection and quantification is the polymerase chain reaction (PCR) (35). PCR amplifies selected NA sequences exponentially through repetitive cycles of DNA denaturation, primer annealing and polymerization (35, 36). This procedure is repeated until a sufficient amount of amplification product is generated that can be visualized by fluorescent intercalating dyes, among others. In real-time or quantitative PCR (qPCR), the amount of generated amplification product is monitored after each amplification cycle, allowing for a more precise quantification of nucleic acids based on the number of cycles needed to overcome a determined detection threshold (37, 38). Addition of molecular beacons (39) and Taqman probes (40) allow for simultaneous quantification and sequence discrimination.

A multitude of alternative amplification techniques has emerged, such as nucleic acid sequence-based amplification (NASBA) (41), strand displacement amplification (SDA) (42, 43), loop-mediated isothermal amplification (LAMP) (44), multiple displacement amplification (MDA) (45) and rolling

circle amplification (RCA) (46, 47), and its modifications hyperbranched RCA (hRCA) (47) and circle-to-circle amplification (48). Most of these amplification methods have the advantage that amplification takes place continuously under isothermal conditions which may facilitate their application in point-of-care (POC) diagnostic tests.

The disadvantage of these methods, except for RCA, is that amplification products constitute single- and double stranded intermediates and diffuse away from the originally detected molecule. Quantification, hence, entirely relies on measuring the overall increase of amplification products. In order to overcome this limitation and enable more precise molecule quantification, a number of pre-compartmentalization strategies have been developed that enable digital quantification, as discussed in detail in the next chapter.

Probes for ligation- and polymerization-based NA detection

Targeted NA analysis is enabled by nucleic acid probes that are specifically designed to recognize a distinct sequence with high specificity and convert this recognition event into a detectable signal. During my thesis work, I have mostly made use of two DNA probing principles whose sequence recognition specificity is based on hybridization and ligation, generating templates for a polymerization reaction that can convert the specific target recognition event into detectable signals.

Padlock probes

Padlock probes comprise two target-complementary arms and a non-hybridizing backbone (34). The target-complementary ends hybridize to their target sequences in juxtaposed position forming a circle on the target sequence that can be enzymatically linked through DNA ligation (34) (figure 1a). The ligation event is strictly target-dependent, rendering the detection highly specific and permitting discrimination of single base substitutions (34, 49). Padlock probes are usually designed to comprise 15-20 nucleotides long target complementary arms, depending on the desired application-dependent melting temperature (T_m). The backbone sequence comprises around 40-50 nucleotides that can be designed to carry a variety of functionalities, as will be discussed later. Circularized padlock probes can be amplified through RCA (50), which will be discussed in detail in the next chapter.

A related probing concept, so called gap-fill padlock probes, or molecular inversion probes (MIPs), hybridize on their target sequence such that a gap is left between both arms which can be filled through polymerization and subsequent ligation, allowing highly specific mutation screening in high multiplex (51).

Selector probes

Instead of circularizing pre-designed synthetic probes on a target sequence, the biological target sequence itself can be circularized. A generic approach is to ligate single stranded DNA fragment ends (52, 53). In a targeted alternative, selector probes specifically circularize selected DNA fragments after restriction digestion of genomic DNA (54, 55) (figure 1b). Selector probes can be designed to directly join the ends of the target fragments (figure 1b) or can introduce vector sequences that can be used for PCR-amplification and hybridization of sequencing anchor probes (56, 57). Alternatively, selected targets can be enriched through MDA which reduces the amplification bias compared to PCR amplification (57). Selector probes have proven highly useful for the targeted enrichment and deep-sequencing of cancer hotspot genes (57-59). The advantage is that sequences with regions of interest, such as cancer hotspot genes, can be circularized and analyzed independent of the probing event.

Selector probe mediated target DNA circularization events can also serve as circular templates for RCA to directly generate sequencing substrates for NGS, a strategy I have explored in my thesis.

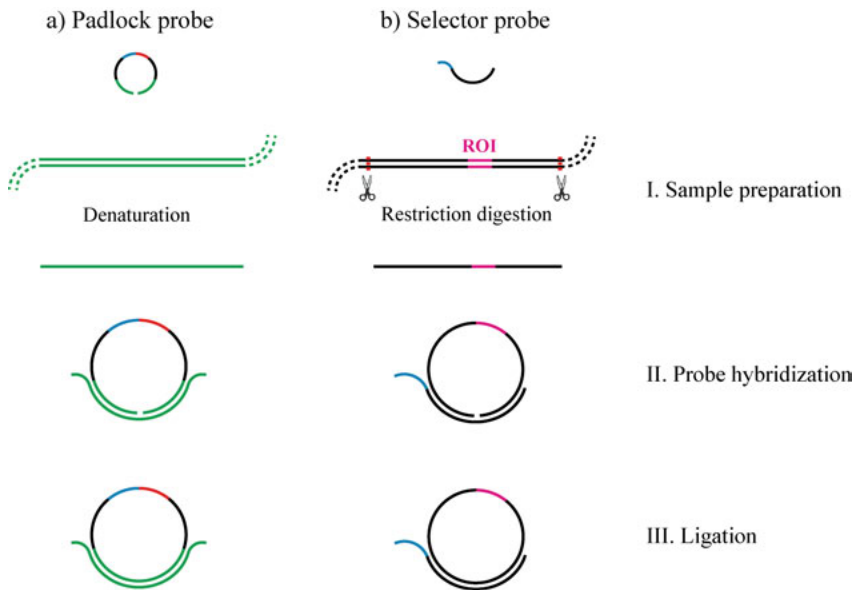


Figure 1: (a) Padlock probes hybridize with their target sequence complementary probe arms on a NA target strand. When perfectly matched the probe arms are enzymatically joined through DNA ligation. Padlock probe backbones can carry sequences with distinct functionality, such as for detection (illustrated in red and blue). (b) Selector probes circularize selected DNA fragments, after enzymatic restriction digestion of genomic DNA, through specific hybridization of the fragment ends on the selector probe template. The DNA fragment ends are joined through ligation. As such, sequences with regions of interest (ROI, illustrated in pink) can be circularized and analyzed independent of the probing event.

Single molecule quantification methods

Some very commonly used diagnostic tests merely require a ‘Yes or No’ answer, e.g., pregnancy tests. The precise concentration of the biomarker indicating pregnancy is not relevant, since the aim is not measuring how much a person is pregnant. Other diagnostic tests, however, require a more quantitative assessment of a certain marker, for example the quantity of glucose in the blood of a diabetic person. It is not sufficient to know that there is glucose in the blood, it is important to know how much. Cancer diagnostic tests require an even more precise measurement of certain biomarker concentrations. In bulk (analog) measurements, reporter molecules, or DNA amplification products, diffuse and dilute into the larger analysis volume. At low target concentrations, the concentration of reporter molecules does not substantially change the overall concentration of the bulk volume and can hardly be detected (figure 2b, upper row). This results in poor sensitivity. Conversely, at elevated target concentrations, the amount of reporter/amplification products quickly saturates and impedes quantification.

“As analytical chemists, the highest resolution measurement one can make is at the single molecule level; it just does not get any better than that.” (David Walt, 2012 (60)). In order to conduct high precision measurements and detect low amounts of target molecules, these molecules must be visualized individually. To this end, a range of different approaches were demonstrated both for *in situ* measurements and solution samples. I will divide these approaches into hybridization- and polymerization- based single molecule quantification methods.

Hybridization-based single molecule quantification

In order to preserve the single molecule information of a detected NA, complementary detection probes, labeled with reporter molecules, such as fluorophores, can be hybridized to NA and resolved through high resolution optical detection. A method for multiplexed amplification-free NA quantification from solute samples, with increasing popularity, is the nanostring nCounter technology (61). Reporter probes with multiple fluorescent dyes in altering sequence are used as optical barcodes that identify distinct NA species. Target NA molecules and hybridized probes are immobilized and electrophoretically stretched out in order to resolve the barcode sequence (61). Alternatively, several detection probes with single fluorophores can be accumulated at the location of the detected molecule, directed through hybridization.

One way to count individual NA molecules *in situ* is single molecule fluorescence *in situ* hybridization (smFISH). Several short sequence-complementary fluorescently labeled oligonucleotides hybridize along the targeted RNA molecule. The spatial increase of fluorescently labeled reporter probes leads to a spatially increased fluorophore concentration appearing as a single dot in high magnification microscopy, reflecting successful hybridization of a single NA molecule (62, 63). Technologies for multiplexed smFISH assays were recently demonstrated (64, 65). However, the throughput of these methods is currently limited by the requirement for high magnification imaging. An approach to achieve a stronger fluorescent signal, and hence allow for faster imaging, is provided by the branched DNA (bDNA) technology in which NAs are hybridized with two to three target-specific primary probes and subsequent hybridization of reporter DNA probes that are labeled with a multitude of fluorophores leading to a strong signal increase (66, 67). This technology can be applied to visualize individual NA molecules *in situ* (67) and in solution (66, 68). Another hybridization-based technique is based on hybridization chain reaction (HCR). Here, a target-specific hybridization probe initiates a chain reaction of localized detection probe hybridization events (69).

Multiplexing and the detection of single nucleotide variations with hybridization-based methods still remain challenging. For these purposes, enzymatic reactions prove more suitable.

Enzymatic single molecule quantification

David Walt pioneered digital ELISA based on the capture of anti-protein-secondary antibody complexes on beads that are subsequently arranged on micro-well arrays, fitting exactly one bead per well. Due to the ultra-small micro-well reaction volume (femto-picoliters), the enzymatic reaction of a single reporter enzyme accumulates sufficient amounts of fluorescent reporter molecules which color those micro-wells that harbor a bead with an individual target protein (70, 71). Digitalization converts a single bulk/analog measurement into many small binary measurements on single molecule level. Digital measurements confine (digitalize) individual target molecules into a multitude of separate reaction vessels in which the same reaction takes place (figure 2a), only the amplification products cannot diffuse, dilute or affect other amplification reactions in any way. By simply counting all ‘positive’ micro-vessels one can count individual molecules (Figure 2b, lower row), which gives a more precise picture of and enables quantification of much lower analyte concentrations.

Similarly, and in fact before the development of digital protein measurements, DNA amplifications were introduced. Already in 1992, the first digital PCR was reported by Sykes, *et al.* when DNA samples were subjected to

limited dilution in microtiter well plates and analyzed using Poisson statistics (72). The Vogelstein lab further developed digital PCR and proved its suitability for quantification of rare mutations in a large background of wild-type sequences (73). The same lab later developed BEAMing (beads, emulsion, amplification and magnetics), a digital PCR technology based on emulsion PCR that enables detection of mutation rates to 0.01% (74). Other compartmentalization strategies (Figure 2a) include dilution and microfluidic separation in micro-chambers (75, 76) and in pico-nanoliter sized droplets (77, 78), as commercialized by Fluidigm and BioRad, respectively. The fundamental difference between digital PCR and qPCR is that digital PCR quantitates the number of initial DNA targets present in a sample, whereas qPCR quantitates PCR products. Separated amplification of molecules reduces amplification bias, increases sensitivity, precision and quantification range (60, 70, 72-74).

Clonal amplification for NGS

It should be noted at this point, that basically all NGS technologies, too, rely on digitalization of amplification reactions in order to create clonal amplicons of single molecules, which can then be sequenced in parallel. Emulsion PCR is used for clonal single DNA molecule amplification on beads in emulsions, used in SoliD, semiconductor (ion torrent), and in pyrosequencing technologies. Bridge PCR amplifies single molecules on a two dimensional surface with PCR, while keeping the amplification product tied to the original location enabling clonal formation of sequencing clusters (7), used in most of Illuminas SBS instruments. RCA generates clonal sequencing substrates by amplifying circularized DNA molecules on a surface (15) or in solution (16).

Most digital compartmentalization and quantification methods require sophisticated technology with complex surface engineering or fluidic systems and instruments. In contrast, in RCA the amplification product is concatemerized, thus preserving its single-molecule integrity. In contrast to all other described digital amplification methods, RCA does not require pre-compartmentalization and can amplify a multitude of single molecules in homogenous solution in parallel (16, 48, 50) (figure 2a).

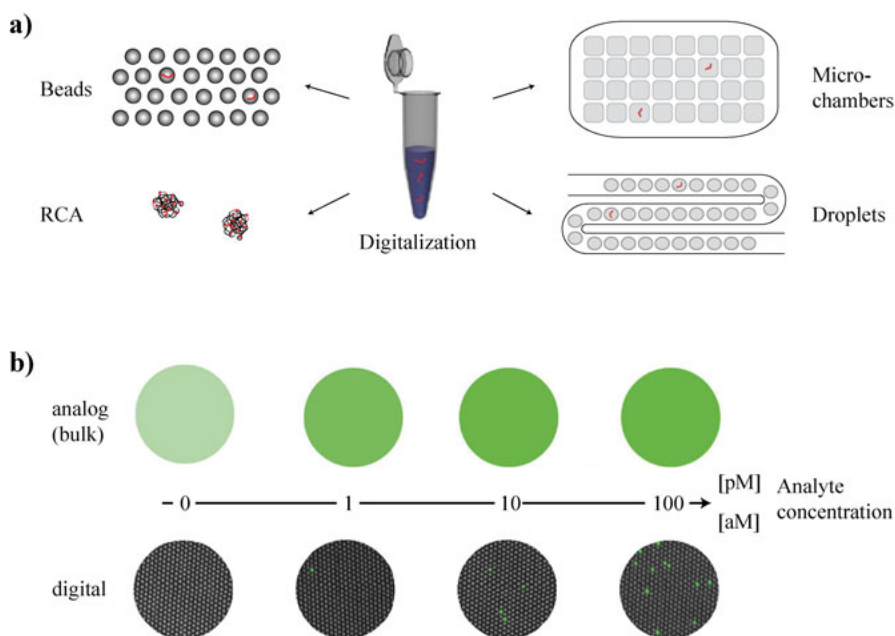


Figure 2: Digitalization of single molecule amplification reactions. (a) Compartmentalization strategies for isolation of single molecules into separate micro-compartments prior to amplification. RCA does not require pre-compartmentalization. (b) Comparison of bulk/analog enzymatic reactions (top row) and digitally compartmentalized reactions (bottom row) over a range of different analyte concentrations. (Figure 2b adopted from (60))

Rolling circle amplification

Since the early 2000's RCA has become an increasingly popular tool for both nucleic acid and protein analysis by supplying a means to clonally amplify a recognized single molecule preserving its single molecule integrity and sequence information. Moreover, when applied *in situ*, the spatial location within the sample is preserved (79, 80). When using phi29 DNA polymerase, a polymerization rate of approximately 1500 nucleotides per minute can be achieved (50). Hence, a 100 nucleotide long circular target can approximately be copied 900 times in one hour RCA reaction time (50). The amplification product consists of hundreds of concatenated circular template copies that collapse into a ~800 nanometer-sized DNA coil. RCA products (RCPs) of padlock probes can be labeled with hundreds identical short fluorescently labeled oligonucleotides that hybridize to the repetitive detection tag sequence introduced by the padlock probe backbone (figure 1a and figure 3) (47, 81). Originating from a single detected NA molecule, the RCA product represents an amplified single molecule which can be digitally quantified directly in solution (81-84) or after deposition on a microscope slide (85).

Due to the nature of DNA comprising a negatively charged phosphate backbone, RCA products are negatively charged which prevents agglomeration and secures counting of individual RCA products (81-83). The specificity provided by the ligation and the isothermal nature of RCA make it an attractive method for application in molecular diagnostic assays. Applications range from diagnosis of infectious diseases to cancer. For example, multiplexed detection of bacterial, fungal and viral pathogens with high specificity were demonstrated (84, 86-93). Padlock probe cocktails with partially degenerated probe-arm sequences can be used to cover variable regions in fast mutating viruses (88, 91). The single base discrimination capability can also be employed to detect bacterial drug-resistance genes (90).

In situ applications range from mutation analysis in cells and directly in preserved tissue (79, 94-96), DNA-protein interactions (97, 98), and gene expression profiling (21).

Through the non-hybridizing backbone, a variety of different functions can be introduced into RCA products making this mode of amplification greatly versatile. Sequence functions can comprise restriction enzyme recognition for RCP monomerization (34, 99), aptamer sequences for protein binding into RCPs (100), compaction for increased RCP integrity, signal-to-noise ratios (101) and RCP detection. Applicable labels for detection probes range from fluorophores (47, 50), quantum dots (102), magnetic particles (103, 104), gold or silver nanoparticles (105) and enzymes (88). The choice of label depends on the sensing principle deployed for read-out. Despite advantages over other digital nucleic acid quantification techniques, the sensitivity of RCA quantification methods has so far greatly been limited by the sensitivity and throughput of the read-out methods that were used. These methods usually detect only a fraction of RCPs within a sample. To compensate for this lack in detection efficiency, circle-to-circle amplification has been developed, which further amplifies RCPs by an intermediate digestion and a second RCA step.

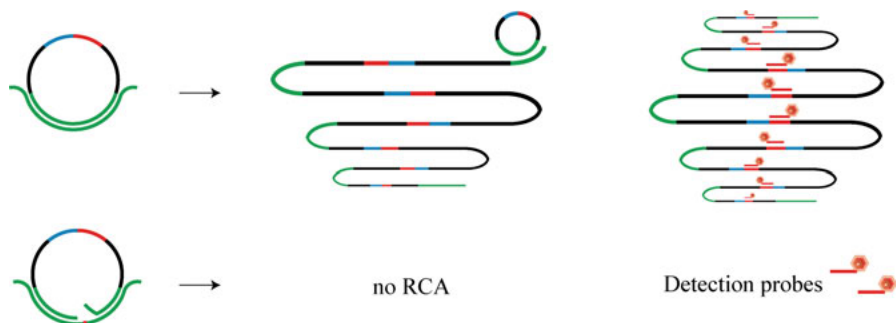


Figure 3: Target-primed rolling circle amplification of padlock probes. Ligated padlock probes can be amplified through RCA, resulting in hundreds of concatenated padlock probe copies. Mismatching probes are not ligated and are not amplified through RCA. Distinct sequences in the backbone of the padlock probe can be used for, e.g. detection purposes. For that purpose, fluorescently labeled detection probes hybridize to the RCA product.

Circle-to-circle amplification

The sensitivity of digital single molecule detection assays, including RCA, entirely relies on the efficiency and throughput of the detection method used for quantification. Single-molecule detection poses the challenge that every generated RCA product has to be detected. As reviewed in the last section of this thesis, most so far reported RCA detection methods only detect a fraction of RCPs in a sample. One way to circumvent the need for the detection of all amplification products is to increase the number of amplification products per detected molecule. In circle-to-circle amplification (C2CA), the generated RCA products are enzymatically digested into monomers by hybridizing oligonucleotides to the restriction site introduced through the backbone sequence of a padlock probe (34, 48, 99), generating a double-stranded restriction site for a restriction enzyme (figure 4). After digestion, the monomers can be re-circularized and ligated on the non-cleaved restriction oligonucleotides and amplified in a second round of RCA (48) (figure 4). The number of generated C2CA products depends on the number of padlock repetitions from the first RCA. In a one hour RCA reaction, approximately 1000 padlock probe copies (90 nucleotides long) are generated (50). Consequently, using C2CA with one hour reaction time in the first RCA, one thousand C2CA products can be generated from a single initially detected molecule (48). Hence, only 0.1% of all C2CA products have to be detected in order to detect one single molecule, substantially reducing the demand on the analysis system.

C2CA facilitates highly multiplexed amplification with little cross-reactivity and less amplification bias compared to PCR (48). Moreover, it was shown

to have a lower variation coefficient (CV) than PCR and is less affected by product inhibition, enabling production of higher amplicon concentrations (48). Despite enabling single-molecule detection, C2CA is a fairly complex assay due to multiple consecutive manual reaction steps. Therefore, automation of C2CA is an important task in order to facilitate the use of C2CA in other laboratories and in diagnostic applications.

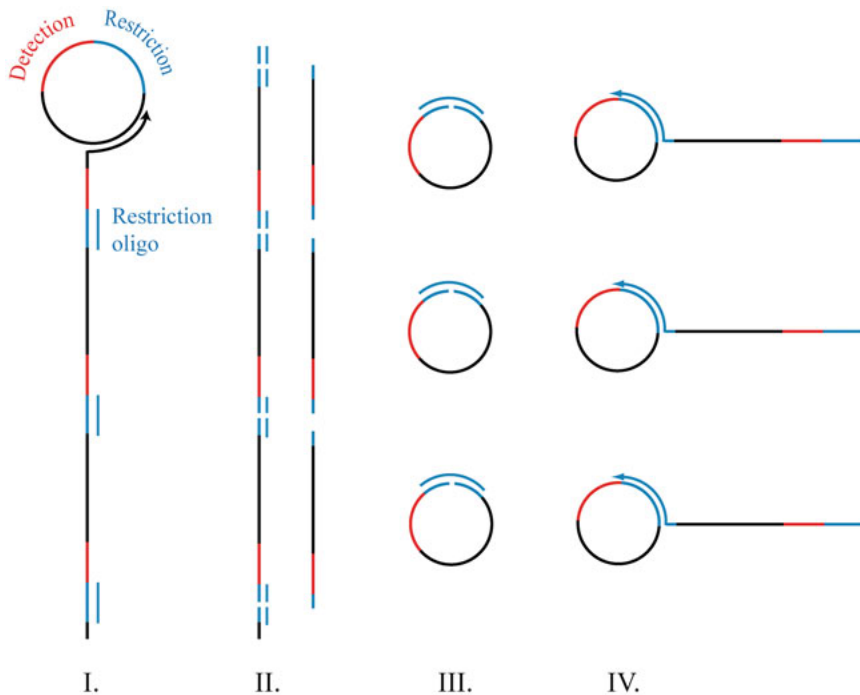


Figure 4: Circle-to-circle amplification (C2CA). (I.) A circle (e.g. a ligated padlock probe) is amplified in a first round of RCA. (II.) A restriction oligonucleotide is annealed to the restriction sequence in the RCA product generating a double stranded restriction site for enzymatic cleavage. (III.) The monomers circularize on uncleaved restriction oligonucleotides and (IV.) are ligated and amplified in a second RCA step.

Magnetic particles in RCA assays

Magnetic particles have proved utterly useful in *in vitro* biomolecular detection assays (106, 107). They provide (i) high analyte capture efficiencies, (ii) flexible broad surface functionalization capability, (iii) a solid support for analyte enrichment and washing, (iv) a means to stir liquids on small scale,

e.g. cocktails, which are preferably stirred instead of shaken, and (v) sensing properties allowing particle-based assay read-out.

In applications that require high assay speed, such as infectious disease diagnostics, padlock probe assays require high probe concentrations in order to rapidly ligate probes on targets. However, high concentrations of excess padlock probes interfere with the RCA reaction. Magnetic particles proved useful for padlock-RCA assays by providing a solid support, which allows rapid NA target capture and removal of excess probes (88, 89). For that purpose, magnetic particles can simply be coupled with target-specific capture oligonucleotides, most conveniently by biotin-streptavidin binding (88, 89). The capture oligonucleotide hybridizes to the target DNA 5'-downstream of the padlock probe. After coupling the complex of capture probe, target and ligated padlock probe to the particle, excess padlock probes can be removed by magnetic washing. Subsequently, RCA can be performed directly on the particle (figure 5). In C2CA, RCA products are monomerized directly on the particles and monomers diffuse into solution, and magnetic particles can be removed. Subsequent C2CA steps are then performed in solution. The use of magnetic particles in C2CA assays significantly reduces the assay time by enabling the use of high padlock probe and ligase concentrations, which reduces the ligation time from several hours to just minutes.

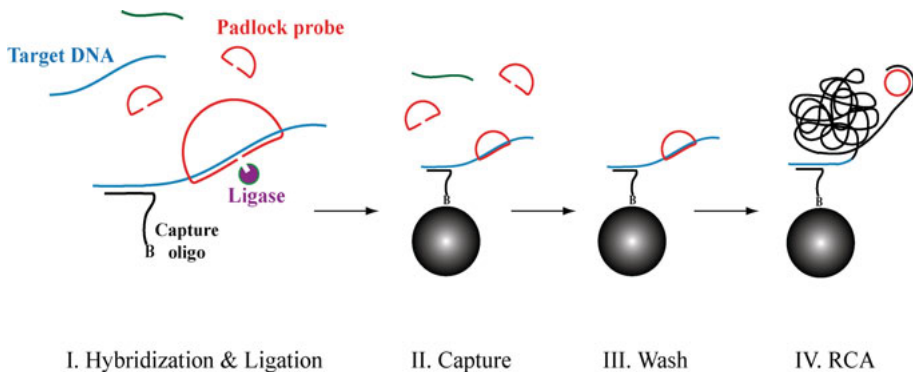


Figure 5: Magnetic particle-based rolling circle amplification assay. (I.) Padlock probes in high concentration are ligated on target DNA. A capture oligonucleotide with a biotin modification hybridizes to the target DNA and (II.) can be captured through magnetic particles. (III.) During magnetic washing, excess padlock probes and sample remains are removed. (IV.) Padlock probes can be amplified through RCA on the particle.

Assay automation

The automation of bio-assays has several benefits both for research and diagnostic applications: (i) Sample processing can be parallelized allowing higher throughput, (ii) Assay precision often increases through automated liquid handling, (iii) the use of smaller reaction volumes reduce the assay cost, and (iv) reduction of manual pipetting labor. Approaches for automation range from large-scale high-throughput robotic pipetting to microfluidic assay miniaturization. The choice depends on the application. High-throughput pipetting robots are often applied in centralized diagnostic laboratories or research facilities to process as many samples in parallel as possible. Microfluidic automation is increasingly applied for sample preparation schemes that require processing of very small reaction volumes and in modern point-of-care diagnostic test devices. It should be mentioned that some of the key components in large-scale high-throughput instruments, such as NGS machines, are based on microfluidics.

Microfluidics

Since the demonstration of the first miniaturized total analysis system (μ TAS) (108, 109), microfluidic systems were increasingly applied to perform laboratory methods, such as biomolecule detection assays, in small-scale, reducing costs and time, as reviewed in great detail (110, 111). In fact, microfluidics has not only facilitated biological research, but also enabled it: It provides a means to accurately handle a multitude of ultra-small reaction volumes in parallel (76). Such small reaction volumes are required, for example, in sample preparation schemes in single-cell proteomics (112) and transcriptomics (113). The same reaction volumes would not be possible to process with standard liquid-handling schemes.

Due to the capability to integrate a range of complex sample processing operations in miniaturized format, microfluidic systems, also referred to as lab-on-chip (LOC) technologies, hold great promise for the future of point-of-care diagnostic tests (111). Microfluidics can generally be divided into three classes comprising (i) continuous-flow microfluidics, (ii) droplet microfluidics and (iii) digital microfluidics. Droplet microfluidics is mainly applied for high-throughput compartmentalization of a reaction, for example for digital PCR, as described above. I will focus on, and briefly describe the microfluidic principles that I made most use of during my thesis work, namely continuous-flow and digital microfluidics (figure 6).

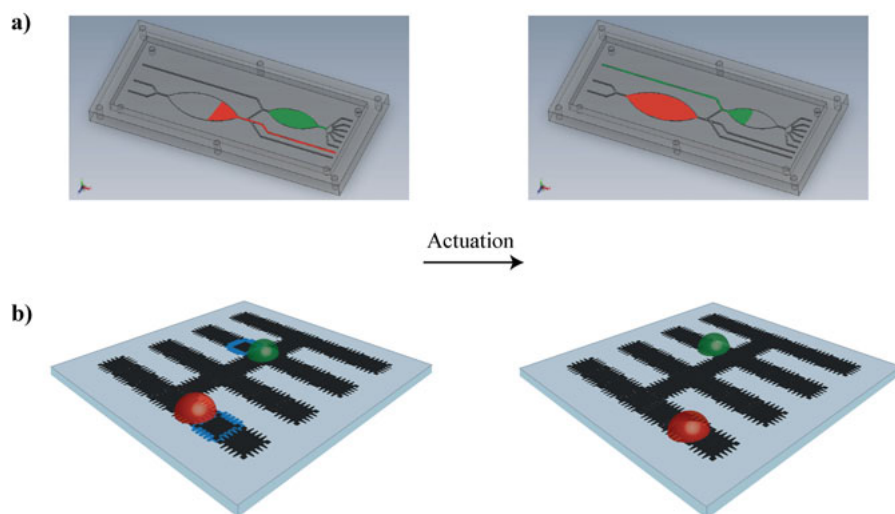


Figure 6: Compartmentalization of reactions and liquid transport in (a) continuous-flow microfluidics and (b) digital microfluidics. (a) Reaction volumes are spatially separated through micro-channels and –chambers. Transport of liquids between channels and chambers is mostly performed through pump pressure. (b) In digital microfluidics reaction volumes are compartmentalized in droplets which can be moved on top of an array of electrodes through the electro-wetting-on-dielectric principle.

Continuous-flow microfluidics

The semiconductor and electronics industry has developed fabrication procedures for micro-structures on small areas, such as computer chips. These procedures were soon adapted to the fabrication of micro-channels and microfluidic chips (108, 109). One of the probably most impactful contributions for microfluidics was the demonstration of poly(di-methyl siloxane) (PDMS) as a simple prototyping material for microfluidic structures and chips (114). This has opened up for the microfluidic integration of a range of biomolecular and chemical laboratory methods.

The performance of bio-assays, such as PCR, in miniaturized format not only decreases cost, but can even increase reaction speed. This can be attributed to a decrease in diffusion distances and faster temperature control (76, 115). Moreover, the automation of liquid suspension and control tends to increase assay robustness by eliminating manual pipetting errors (76). The most common way of liquid handling is flow in separate micro-channels and –chambers, which provides the compartmentalization necessary to perform separate consecutive steps (figure 6a). Reactions can take place in solution or on the surface of reaction chambers. A variety of actuating mechanisms, such as micro-valves and –mixers, enable compartmentalization of reactions

into a multitude of individually addressable reaction chambers (75, 76). Moreover, the use of micro-pillars (116), magnetic particles (107) and membranes (117) has enabled the specific capture and subsequent analysis of biomolecules. Recently, a microfluidic analysis system was proposed for microfluidic membrane-based DNA concentration and subsequent on-chip PCR analysis (118). We used a similar concept for high-efficiency detection of RCA products, described during the present investigations in this thesis.

Although fabrication of continuous-flow microfluidic chips is relatively simple, it requires much external equipment, such as pumps, external valve controls, tubing, etc., to control liquids on chip. The prototype stage usually resembles a 'chip-in-a-lab' rather than a 'lab-on-a-chip'. Moreover, the use of magnetic particles in multistep assays on continuous flow chips can be complex. Inefficient particle mixing, especially in RCA-based assays, negatively affects the assay efficiency (unpublished data). For this purpose the use of the digital microfluidic principle is advantageous.

Digital microfluidics

An alternative microfluidic liquid compartmentalization and handling principle is digital microfluidics (DMF). Liquids are applied onto a DMF chip in nL - μ L volumes and naturally form droplets on the hydrophobic chip surface. A DMF chip comprises an array of mm-sized electrodes, which can be actuated individually, and an insulating hydrophobic layer. The controlled transportation of droplets on chip is facilitated through the electrowetting-on-dielectric (EWOD) principle (119). In brief, by actuation of electrodes under the hydrophobic layer, the surface is locally rendered electrostatic. As a consequence, the contact angle of a droplet that is touching this activated area changes and the droplet moves to the activated surface area (119-121) (figure 6b). Exploiting this phenomenon, droplets carrying different reaction mixtures can be addressed individually, and transported along an array of individually actuatable electrodes. Droplets can be merged, mixed and split (121, 122). The versatility of this mode of microfluidic liquid handling allows for the performance of complex multistep bioassays (121-125).

Interestingly, it was shown that the movement of droplets on DMF chips leads to very effective mixing due to an internal vortex created by the droplet movement (126, 127). This gives DMF a major advantage for performing magnetic particle-based assays. As such, magnetic particles can be separated, washed, mixed and incubated with new reagents, which facilitates automation of complex multistep solid-phase assays (122, 123, 128, 129). A relevant example for an application of DMF for the automation of complex multistep assays is the automated library preparation of several samples in

parallel for next generation sequencing, performed on a DMF platform from Liquid logic, now acquired by Illumina.

DMF should not be confused with droplet microfluidics, which is occasionally named digital microfluidics due to the great capability to generate individual single-molecule or single cell reactions in millions of droplets. The capability to execute consecutive reaction steps and other droplet manipulations is, however, strongly limited in droplet microfluidics, making it less applicable for multistep assay automation.

Single molecule read-out

The sensitivity of a molecular detection method, including all digital single molecule assays, depends on the capacity of the read-out method. In digital droplet PCR, for instance, a relatively large dilution has to be applied to the sample to ensure that not more than one molecule is encapsulated in one droplet, resulting in a large number of droplets staying empty. To detect the few 'positive' droplets among all droplets, a large fraction or even all droplets have to be analyzed, when aiming to detect every single molecule. This requires a detector system with high throughput, accuracy and sensitivity. Similarly, RCA assays require detection of a large fraction of RCA products in order to obtain high sensitivity. Digital read-out of RCPs, representing amplified single molecules (ASMs), enables high sensitivity and precision.

Digital RCA read out

In principle, digital RCA read-out approaches can be divided into two main categories: (i) surface-attached read-out, in which RCPs are either directly generated on a surface, or first generated in solution and then subsequently transferred to a surface, (ii) homogenous read-out, in which RCPs are directly quantified in solution.

Surface-attached read-out is probably easiest to adopt for RCP read-out. NGS of RCP substrates can be placed into the same category (15, 16). However, in order to analyze every RCP the entire surface area has to be imaged, requiring more time and sophisticated imaging equipment.

Homogenous RCA read-out relies on sensing RCPs in movement (81, 82). RCPs can be quantified, for example, by passing RCP solutions through a focal plane of a confocal microscope while flowing through a microfluidic channel (84, 130). Due to the relatively large channel dimensions required for fast flow rate, as well as the narrow confocal volume, only 0.1-1% of RCPs are imaged (84). The detection efficiency was later increased to approximately 1-2% (89). Further increase in the detection efficiency was at-

tempted by locally enriching RCPs in micro-channels patterned with capture oligonucleotides (131), or filled with capturing beads (132). However, a substantial increase in detection efficiency could not be demonstrated.

A major objective of my thesis was to explore alternative RCP sensing and quantification principles, potentially yielding higher detection efficiencies and simplifying the read-out. For surface-attached RCP quantification, the feasibility of simple low-cost imaging equipment, such as that in modern smart phone cameras was investigated (133). For homogeneous RCP detection, the resistive pulse sensing (RPS) principle was investigated. RPS enables quantification of individual objects in solution, but has not yet been explored as RCA read-out.

Resistive pulse sensing

In the 1950s, Coulter proposed a principle that permits detection of single cells or other objects in micrometer scale, based on the blockade of an ion current that can be created by applying a voltage between two compartments that are connected through a slim channel. Objects that pass through the channel from one to the other compartment temporarily block the flow of ions through the channel which can be detected as a drop in current (134). This principle is called resistive pulse sensing (RPS) and is still commonly used for cell counting and size determination in the Coulter counter (134).

Recent advances in nano- and micro-engineering enabled the precise fabrication of solid-state nano- and micropores that allow for RPS measurements of smaller objects (17). With advances in the sensing technology more parameters of the objects can be analyzed (135) (figure 7). Concentration measurements are enabled by measuring the frequency of blockade events. Precise size distributions of particles in a sample can be obtained from the blockade magnitude of measured particles (136). The passage time of a particle through the pore, as determined by the blockade duration, gives an indication about the particle shape or charge (135, 136). Tunable RPS nanopores have been used for the direct detection and size characterization of DNA and of particles modified with DNA (136, 137). The passage time of DNA-modified particles through a pore is prolonged compared to unmodified particles (136, 138).

Nanopore RPS has been used early for the detection of individual proteins and nucleic acids (17, 19, 139). Nanopores that are capable of reading the base composition of a single-stranded DNA strand are under heavy development and in the early phase of commercialization (Oxford nanopores). The sequencing principle is based on the unique current blockade signatures of the individual bases (17-19). DNA sequencing nanopores have diameters

of around 3-5 nanometer. In this thesis, I made use of larger pores for the quantification of particles and RCPs in the scale 100 nm – 2 μ m.

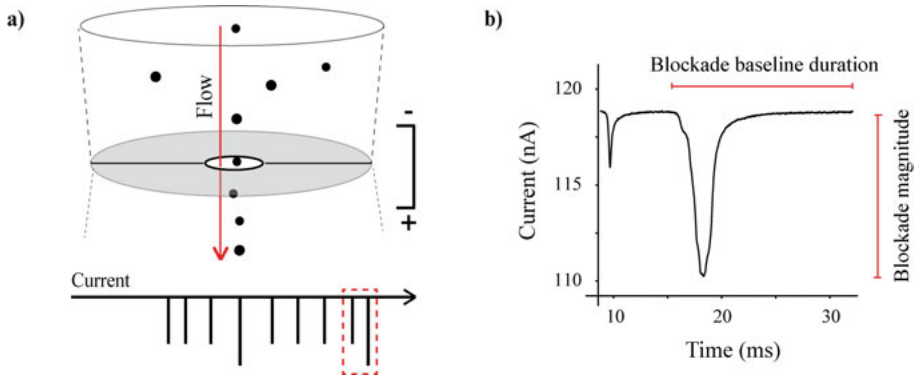


Figure 7: Resistive pulse sensing principle. (a) Microfluidic flow of particles from one compartment to the other through a RPS nano- or micropore. An applied voltage between the compartments induces an ion current that is temporarily blocked by trans-passing particles. (b) Blow up of the two blockade signals marked in red dashed box in a. Blockade magnitude and blockade baseline duration indicate relative particle size, and charge, respectively.

Present Investigations

Paper I. Circle-to-circle amplification on a digital microfluidic chip for amplified single molecule detection

Background

DNA detection with padlock probes and subsequent amplification with Circle-to-circle amplification (C2CA) enables DNA detection with high specificity and sensitivity. Despite several advantages of C2CA over other DNA amplification schemes, one major drawback is the relatively high assay complexity, generated by the number of sequential steps. In order to bring the advantages of RCA and C2CA assays to diagnostic application, the assay has to be integrated and automated in a fluidic system. Compared to large robotic systems, assay automation on microfluidic chips offers several advantages, i.e. decreased reagent consumption and device cost.

In a previously described effort we aimed to integrate C2CA in a continuous-flow microfluidic chip fabricated with a novel material that holds great promises for bio-compatibility (140). Promising results were obtained for on-chip ligation and RCA, but the consecutive performance of both assay steps resulted in strong decrease in reaction efficiency due to inefficient mixing of magnetic particles. The movement of droplets by electrowetting-on-dielectric on digital microfluidic (DMF) chips leads to effective mixing inside the droplet (126, 127). Moreover, droplets on DMF can be used as individually addressable reaction compartments that can be merged and split in order to perform assay steps (129). In this paper, in collaboration with the Lammertyn lab in Belgium, we made use of these unique properties and integrated C2CA on a DMF chip.

Summary

In order to facilitate the performance of C2CA on a DMF chip, several parameters had to be optimized, such as the on-chip movability of reaction mixes. Moreover, a new magnetic particle extraction and -transfer procedure was developed that allowed for particle extraction with near 100% efficiency

and the shuttling of particles between droplets, which facilitated whole C2CA assay performance on the DMF chip. We tested the individual enzymatic reaction steps of C2CA on the DMF chip and found stable enzyme reaction efficiencies on-chip compared to reaction tube conditions. We then performed the complete C2CA assay with all sequential steps and achieved nearly 100% assay efficiency compared to standard tube reaction conditions. C2CA products were quantified by standard microscope slide quantification. A limit of detection of 1 aM DNA concentration was achieved on-chip with a dynamic range of 4 orders of magnitude up to 10 fM. Conclusively, we demonstrated how C2CA can be performed on a DMF platform with high assay efficiency.

Discussion

This was the first time that C2CA was performed integrated on a microfluidic chip with nearly 100% reaction efficiency as compared to standard tube conditions. The compactness of DMF, with no further fluidic pumps or tubing necessary and all actuation controls integrated in one circuit board, makes DMF-C2CA a promising combination for the development of automated C2CA devices in bench-top or portable format. In this study, we proved the concept for integration and provided the necessary parameters for total automation. Because a suitable technology to heat-inactivate enzymes in droplets was not available at the time, we manually inserted reaction mixtures consecutively. Moreover, extraction and transfer for magnetic beads between droplets required manual positioning. The integration of a micro-heater element and a controllable magnet positioner will enable complete automation. Additionally, the integration of the read-out on-chip should be investigated which may pave the way towards a total integrated C2CA analysis system.

Paper II. Digital quantification of rolling circle amplified single DNA molecules in a resistive pulse sensing nanopore

Background

Digital quantification of RCA products offers the highest quantification precision and sensitivity to RCA-based assays. The sensitivity, however, is limited by the digital detection method used as read-out. The methods reported so far only count a fraction of RCPs, because (i) the throughput of the detection method allows analyzing only part of the sample volume, or (ii) the detector only senses a fraction of RCPs within the entire sample volume. Low detection efficiencies are usually compensated for by C2CA, as described in paper I. The added assay complexity, however, demands a high complexity of devices for automated processing and quantification of C2CA. A higher detection efficiency of RCA products, however, would circumvent the need for additional amplification. Employing a sensing principle that recognizes every individual RCP passing by the sensor would increase the detection efficiency. On the microscopic level, RCPs are relatively large bulky objects. A sensing principle that allows counting every individual object passing through a detector is resistive pulse sensing (RPS). It measures a transient blockage of ion current when an object passes through a small pore. The aim of this paper was to investigate a new sensing principle for RCA products based on resistive pulse sensing and to create an assay format suited for the RPS read out of solid-phase RCA assays.

Summary

We present the first RPS measurements of RCA products, directly in solution, and attached to magnetic particles as a solid support. In solution RPS measurements enabled a label-free size characterization of RCPs, which is consistent with previously reported size distributions based on fluorescent measurements. The RPS size characterization is not effected by the point-spread function, which introduces size bias into fluorescent measurements. However, our data also demonstrates problems with RPS measurements of RCPs in solution, i.e. the quantification precision was low and higher concentrations of RCA products could not be measured, probably due to clustering and ‘jamming’ effects. Rigid particles, on the other hand, can be analyzed with very consistent particle flow-rates, because rigid particles interact less with each other, and with the nanopore membrane. We have previously demonstrated that magnetic particles can increase RCA assay speed by providing a means to capture DNA targets faster onto a solid support and

remove excess padlock probes and sample remains. RCA can be performed directly on magnetic particles. We investigated whether RCPs on particles can be sensed by RPS and distinguished from particles without attached RCPs. We found that individual RCPs attached on particles can be discriminated from 'blank' particles based on (i) increased size and (ii) baseline duration of the particle. We set up a digital assay in which a consistent number of particles capture low concentrations of DNA targets. According to Poisson distribution there is a low chance of capturing more than one molecule per particle. After RCA, the particles were analyzed in RPS and particles with attached RCPs quantified. As little as 50 zmol synthetic DNA could be detected. With a total RCA assay time of only 40 min, and an analysis time of 10 min, this method facilitates quantitative DNA detection within a sample in a simple, rapid, sensitive and inexpensive manner.

Discussion

The main motivation of this study was to adopt a sensing principle that allows measuring every single RCP passing through a detector. Homogeneous amplified single molecule detection is limited by measuring only a fraction of RCPs passing through the confocal plane within a microfluidic flow channel. The RPS sensing principle allows detecting every individual RCP passing through the sensor. At higher flow-rates and elevated concentrations, however, the measurements became unreproducible. We speculate that clustering effects and interactions with the nanopore membrane caused these variations. Hence, this method cannot be used to quantify RCA products in solution. On the other hand, we found that particles with single attached RCPs can be distinguished from blank particles. Based on this observation, we developed a new digital quantification assay with RCA products analyzed directly on magnetic particles. We provided a proof-of-concept, and demonstrated a low LOD achieved with a simple four-step assay including a single 20 min RCA amplification step. The digital read-out on particles has a quantification range of three orders of magnitude, which can potentially be extended by the use of more particles and by analysis of more particles. We limited the analysis to 10000 particles which can be analyzed in 10 min. Faster flow-rates might cause 'jamming' problems and would render the measurement less sensitive to RCPs attached on particles. Hence, despite detecting every RCP passing through the sensor, only 1% of particles can be analyzed in relatively short time. In order to extend the throughput, several pores could be parallelized on arrays similar to nanopore sequencing instruments. This sensing principle can be very cost-effective and can be miniaturized on small microfluidic chips, potentially enabling very sensitive digital quantification of RCA assays in portable format. Further research needs to be conducted in order to further characterize and optimize the principle of sensing RCPs in solution or attached on particles using RPS.

Paper III. Microfluidic enrichment and targeted sequencing of rolling circle amplified single molecules

Background

As already mentioned, the sensitivity of RCA assays is strongly limited by the detection method used as read-out. The ideal read-out system for high speed and high sensitivity comprises a detection method that has the capacity to (i) detect individual RCPs, (ii) analyze the entire volume of a sample in relatively short time, and (iii) recognize every RCP within that volume. When deposited on a microscopic slide, RCPs spread out over the large two-dimensional surface under a cover slip (85). At 10-times magnification one field of view only comprises $\sim 0.2\%$ of that area. For high sensitivity the entire area has to be scanned in order to detect low amounts of RCPs. Homogenous amplified single molecule detection quantifies RCPs in solution while flowing through a confocal line scanner (84, 130). However, this method does not detect a higher fraction of RCPs in solutions than the standard microscope slide read-out. A commercial version of this detection system (Aquila 1000, Qlinea) has an increased efficiency of 1-2% (89). Since then, several alternative methods have been proposed for the detection of RCPs (see table 1 in Perspectives). Despite the simplicity of devices, the detection efficiency has not been improved. Even in paper II, the aim was to increase the detection efficiency of RCPs. Although the sensor detects nearly all RCPs passing by, only a fraction of the sample volume could be analyzed.

The aim of this paper was to develop a new RCP quantification method with substantially higher detection efficiency by enrichment of RCPs from large sample volumes onto the sensor plane of a fluorescent imager. Moreover, it was investigated whether enriched RCPs could be sequenced through NGS chemistry.

Summary

A microfluidic chip was fabricated that is capable of concentrating RCA products from larger sample volumes onto a spatially defined small two-dimensional area that can directly be imaged. Hundreds of fields of view on a microscope slide were fluidically reduced into one field of view of a low magnification microscope objective. Microfluidic enrichment led to a very strong increase in detection efficiency, enabling the detection of 5 aM RCP concentration in 50 μL volume. The herein reported microfluidic enrichment method has another main advantage over solution-based quantification methods: enriched RCPs can be further analyzed with NGS chemistry. For a proof-of-concept, we enriched and successfully sequenced an equimolar

mixture of RCPs containing four different barcode sequences directly on chip with sequencing chemistry and integrated imaging. Microfluidic RCP enrichment and sequencing (μ RESEQ) enables the analysis of ultra-low RCP concentrations and allows for multiplexed nucleic acid quantification with simple one-step RCA assays, making additional amplification steps obsolete. Enriched RCPs can directly be sequenced on chip, enabling targeted sequencing analysis of low RCP concentrations.

Discussion

With μ RESEQ, we demonstrated an unprecedented RCA product detection efficiency. This is facilitated through the spatial enrichment of RCPs from larger sample volumes into the focal detector plane, making imaging of RCPs ~ 45 times more efficient than analysis on a standard microscope slide (no enrichment). Previously reported RCP read-out methods only detect a small fraction of RCPs within a sample. In order to detect minute amounts of nucleic acids, additional amplification steps, such as C2CA, have to be introduced into the assay to compensate for low detection efficiencies. Additional amplification steps, however, introduce a lot more assay complexity, which makes application in other laboratories or diagnostic tests less favorable, but also complicates the integration and automation in microfluidic chips. μ RESEQ, on the other hand, enables the use of simple one-step RCA assays with high sensitivity, facilitated through the high detection efficiency. Simple one-step RCA assays with high sensitivity are more applicable in diagnostic tests and make μ RESEQ an ideal read-out platform for RCA-based diagnostic applications. Moreover, μ RESEQ can function as a mini-sequencing platform for targeted DNA sequencing applications. In comparison with most commercially available sequencing instruments, μ RESEQ is set out to sequence a very low amount of sequencing substrates originating from low sample DNA concentrations and targeted library preparations. When sequencing low concentrations of sequencing libraries on commercial instruments, costs per sequenced base become very high due to sparsely filled arrays. Alternatively, low library concentrations are pre-amplified which can introduce amplification biases that can impede the detection of rare mutations (53). μ RESEQ enriches low library concentrations without need for pre-amplification potentially giving higher specificity. μ RESEQ enables low-cost small-scale sequencing and hopefully paves the way for the development of cost-effective small-scale sequencing instruments available to every laboratory.

Paper IV. *In situ* detection of KRAS point mutations and targeted DNA sequencing with a mobile phone

Background

Digital quantification of RCA products gives RCA assays the highest possible quantification precision and sensitivity. On the microscopic level, RCPs are very bright objects when labeled with hundreds of fluorescently tagged detection probes, facilitating imaging with simple low-magnification imaging technology. The Ozcan lab at UCLA has demonstrated that mobile phones can be equipped with simple cost-effective add-ons which convert smart phone cameras into microscopes (133). The incentive of this paper was to investigate the possibility to image and quantify individual RCA products with a modified smart phone camera. Applications for this could be numerous. One possible application is pathologic tumor heterogeneity analysis. Traditionally, morphological tumor biopsy analysis and Gleason scoring takes place at the pathologist's office. Molecular analysis of the tumor biopsies, however, is outsourced to centralized laboratories. RCA facilitates simple *in situ* mutation analysis directly in preserved tumor tissue. A mobile phone powered *in situ* mutation analysis and targeted sequencing imaging device could facilitate molecular analysis directly at the pathologist's office, and at other points-of-care.

Summary

In collaboration with UCLA researchers, a 20-megapixel smart phone camera was converted into a multiplex fluorescent RCP imager. Using this mobile phone driven imager, RCPs were imaged on slides and inside cells and tissues. RCA reaction time and RCP integrity were optimized for mobile phone imaging. Machine learning-based image analysis software was implemented for automated cell and RCP recognition. We performed *in situ* KRAS point mutation assays on KRAS wild type - KRAS mutant cell line mixtures in different ratios. 1% KRAS mutant cells could be distinguished from wild type cells. Finally, we applied the KRAS genotyping assay on clinical tumor samples and succeeded to image and quantify RCPs *in situ* with the mobile phone. Additionally, we developed a simple three-step targeted sequencing sample preparation scheme, optimal for mobile phone-based sequencing, and performed targeted DNA sequencing on extracted cell line DNA to investigate the sequencing specificity. Our sample preparation scheme enables targeted sequencing with ~100x sequencing depth, potentially enabling somatic mutation detection down to ~1%. A heterogeneous KRAS codon 12.2 mutation in the A427 cell line DNA extracts was correctly identified by detecting both wild type and mutant specific sequencing

signals, proving the feasibility of this approach. Conclusively, we demonstrate how a mobile phone with simple low-cost attachment is used for multiplex *in situ* point mutation detection directly in preserved tumor tissue and for targeted DNA sequencing of extracted tumor DNA.

Discussion

The mobile phone imaging based *in situ* point mutation analysis demonstrates the feasibility of a mobile phone powered molecular tissue heterogeneity analysis tool that may provide a simple means to integrating molecular marker information with traditional morphology analysis at the pathologist office. It may also contribute to increasingly enable cancer diagnostics in resource-poor places and may prove useful in other field applications.

Generally, the aim of this study was to investigate a low-cost imaging technology for RCPs, which may be compatible with the μ RESEQ concept presented in paper III. RCPs enriched into one focal plane require a single simple image. While most low-cost RCP detection methods measure in bulk instead of detecting individual RCPs, a simple fluorescent imaging system that can recognize and distinguish individual RCPs would essentially have a similar sensitivity as other digital fluorescent imaging-based quantification methods. Due to the great availability and spread of smart phones we may use the imaging capacity, and potentially the computing capacity, to quantify RCA products and image targeted sequencing reactions. Together this may truly lead to the development of ultra-low-cost RCA-based diagnostic devices, especially at the point-of care and in resource-poor environments.

Perspectives

High precision and sensitivity are decisive parameters for molecular diagnostic tests. In principle, RCA assays offer these parameters. The sensitivity of RCA assays is not limited by the assay itself, but by the detection method that is used. Low detection efficiencies can be compensated for by additional amplification steps, such as through C2CA. The relatively high assay complexity of C2CA, however, renders the application of C2CA in other laboratories and in diagnostic tests less favorable. My first main focus was to automate C2CA on a microfluidic system in order to increase its applicability. Paper I demonstrates the integration of C2CA on a digital microfluidic chip, which proved to be a highly suitable microfluidic system for this assay (141). Through continued development, this could potentially become a fully automated and compact benchtop system.

As the next step, I investigated the integration of RCP read-out on the chip. During this process I realized that the read-out is *the* limiting factor for the sensitivity of an RCA assay. RCA assays are truly digital, i.e., one single molecule is converted into one countable RCP. If we detect all RCPs, or at least a larger fraction, there is no need for further amplification. This is also true for the digital microfluidic chip: if all generated RCPs were detectable on chip, C2CA can be avoided. I have dedicated most of my thesis to the development of more sensitive RCP read-out methods that make simple RCA assays more sensitive.

Since I am not the first to develop alternative RCA read out methods, I compiled an overview of the most so far reported RCA read-out methods and their detection sensitivities. I hope this will give some perspective and help decide how RCA assays should preferably be read out in the future.

RCA read-out methods – a comparison

As discussed during the previous chapters, most digital RCA-based detection methods rely on fluorescence. Fluorescently labeled RCPs can be discriminated from the background, even in homogeneous solution, with high signal to noise ratios (81, 82, 84, 85, 89, 130). Integrated fluorescence-based digital RCA read-out methods were proposed, which integrated DNA detection, RCA amplification and read-out, based on (i) micro-bead filled fluidic channels (132, 142) or (ii) capture probe functionalized patterns in ultra-thin mi-

cro-channels (131). During my thesis I added the concept of sensing RCPs through RPS as an alternative non-fluorescent digital read out (143).

A range of alternative non-digital RCA sensing principles have been proposed that may be more cost-effective and simple. An early fluorescence based alternative read-out was based on real-time quantification of RCA during the reaction using molecular beacons (99). A colorimetric reporter-enzyme based detection method used RCP detection oligonucleotides labeled with HRP. The HRP-catalyzed color change could be detected by eye or through absorbance measurement (88). Another colorimetric read-out uses monomerized RCPs to cluster gold nanoparticles, conjugated to detection oligonucleotides, changing the refractive index, visible to the naked eye (144). One of the most explored non fluorescent RCP sensing principle is the volume amplified magnetic nanobead detection assay (VAMNDA). The presence of RCPs is sensed by the change of magnetic properties of the magnetic labels bound to RCPs (90, 103, 104, 145). A similar concept is based on the opto-magnetic sensing of RCPs by a blue ray laser (93). In this assay format, monomerized RCPs promote the cluster formation of magnetic nanobeads functionalized with complementary oligonucleotides. In a recently demonstrated electrical RCA sensing concept, RCPs are stretched out over two electrodes and metalized through gold nanoparticle labeling and silver enhancement, creating DNA nanowires that close an electrical circuit (146). This concept might open up for very low-cost RCA-based biosensors. Similarly, RCPs were also metalized in coil structure, making RCPs visible in bright field imaging (147).

In the following table, RCA read-out methods with respective performances are summarized, focusing on detection efficiency and sensitivity.

Table 1: Reported RCA read-out methods

Read out method	Analysis time	Multiplexing capability	Sensitivity	Fraction of detectable RCPs	Deeper RCP analysis
Microscope slide (85) ^d , 1 field of view 10x magnification	<1 min	limited by available fluorophores	5 fM (10 μ L)	0.1-1 % (depends on area)	Yes
Microscope slide, whole area ^d	20-120 min	limited by available fluorophores	Single RCP	100 %	Yes
Amplified single molecule detection (ASMD) (84) ^d	3 min	2	5 fM (5 μ L)	0.1 %	No
ASMD, Aquila1000 ^{d*}	4 min	1-2	0.5 fM (50 μ L)	2 %	No
Microbead-RCA in	1-10	limited by	3 fM	0.1-8 %**	No

micro-channels (132) ^d	min**	available fluorophores	(5μL)		
RCA in patterned Micro-channels (131) ^d	1-5 min**	limited by available fluorophores	50 fM (***)	un- known***	potentially
Gel electrophoresis (148, 149)	2 min	~5	25ng vibrio C2CA	***	No
VAMNDA (103, 104)	2 min	1-2	3 pM (50 μL)	0.000001 %	No
Optomagnetic nano- bead (93)		1-2 (but scal- able)	50 fM (32 μL)	0.0005%	No
RCA gold nanowire (146)		1 (but scala- ble)	66 pM (10 μL)	un- known***	No
Gold nanoparticle aggregation (144)	1 min	1	70 fM (60 μL)	0.00004 %	No
Bulk fluorescent measurement (99)	1 min	3-4	20 pM (50 μL)	0.0000002 %	No
Colorimetric HRP read-out (88)		1		0.00002%	No
RPS (143) ^d	10 min	1	250 aM (40μL)	1 %	No
μRESEQ-PC ^d	2 min	limited by available fluorophores	50 aM (50μL)	2 %	Yes
μRESEQ-NC ^d	2 min	limited by available fluorophores	5 aM (50μL)	10-60 % (conc. de- pendent)	Yes

^dDigital read-out

*Aquila 1000, Qlinea, Sweden

**Depending on number of images taken. Not reported.

***Volume unclear. Not reported.

In summary, it is obvious that digital read out methods (marked with ^d) detect a larger fraction of RCPs within a sample, and hence, are superior in sensitivity. The reason for superior sensitivity of digital read-outs is explained in detail in the previous chapters. Another observation that can be made from this comparison is that approaches that concentrate RCPs into smaller analysis volumes or onto smaller imaging areas increase the analysis speed and the sensitivity per analysis time. These approaches make RCA a highly sensitive amplification mode for single molecule detection.

Conclusions and outlook

The future of C2CA

I am not trying to say that C2CA is obsolete. In fact, the opposite is true. What should be reconsidered, though, is what C2CA is used for. With no doubt, C2CA is a powerful amplification mode with several benefits over PCR: the amplification is less sequence biased, being able to amplify GC rich and hairpin structure sequences with great efficiency. Moreover, C2CA is unique in producing single stranded amplicons, making it an optimal mode of production for single stranded probe entities, such as DNA origami tile oligonucleotides and smFISH probes (150). It should potentially prove suitable for the production of single stranded probes in general, e.g. padlock probes, and for other functional oligonucleotides, such as aptamers. For diagnostic application, C2CA can well be applied as means for DNA detection, automated in large-scale, in high-throughput facilities. For POC diagnostic devices, let's be frank, it will most likely not be the underlying molecular technology of choice. Instead, when using a read-out method with high RCP detection capacity, like the herein presented μ RESEQ method, simple one step RCA assays are more suitable for POC diagnostic devices.

The future of RCA read-out

Let's face it: counting individual RCA products is always better than measuring only the approximate amount of DNA produced through RCA. Despite all arguments for increased simplicity, or lower device costs of sensor systems that measure in bulk, counting individual RCPs is better. And here is the spoiler: digital read-out does not have to be expensive. RPS, for example, seems to be a promising sensing concept that can be miniaturized and integrated into very cost-effective portable instruments. Just have a look at the MinION from Oxford nanopores.

Even fluorescent imagers are not necessarily expensive or require large instrumentation. You carry one in your pocket. Both the imaging and the computational capacity of smart phones grows as fast, or even faster than Moore's law (133). Fluorescently labeled RCPs are very bright objects, and have been that bright since the late 1990s. They needed to be bright because back then microscopes would not detect them otherwise. Smart phone imaging technology of today has reached the technological level of microscopes from the 90s (133). With a cheap add-on, everyone can potentially use their own smart phone to image RCPs.

Future RCA read-out should be digital. Let's not motivate more efforts to measure RCA products with non-sensitive sensors. That is nonsense. It is like throwing pearls into water and measure the change of refractive index. Future RCA read-out should and will be digital.

Acknowledgements

A peculiar feeling to sit and write acknowledgments for a time that passed by so fast. It's almost like time-traveling. It has been a very exciting, fun and fulfilling journey. Even after a lot of hard work in the end, I still like my job. Part of this is due to the creative research we are doing in this lab. But a bigger part of that is due to the amazing people that I have had the privilege to work with. And hence, my first gratitude is addressed to my supervisor **Mats Nilsson**. Thank you very much for taking me on as PhD student and for throwing me into the magnetic particle project. You have always been a strong magnet for me. ;) I think you are the most optimistic and enthusiastic person I have met. Thanks for everything. My co-supervisor **Ulf Landegren**, probably the most knowledgeable (in bio-assays, but I am sure you know other stuff, too..) person I know! Thanks for inspiring discussions, your critical opinion, always having time and for an introduction to autumn- after sauna-skinny dipping.

Thanks **Ola**, for interesting discussions and for trying hard to make me join swimming early morning. I would still never do that! **Masood**, for being such an enthusiastic BioMaX-travel partner ;). Thanks **Erik**, for knowing what everyone is doing, for your dry humor, and for funny old stories.

Thanks to the European Commission for funding the **BioMaX** program, which was fantastic. I learned a lot! Thanks to everyone that participated, especially to **Sole, Delfina, Stefano, Fabiola, Iulia, Elena, Tamara, Tonge, Beda, Diego, Matteo, Deniz, Rory** and **Donacien**. Thanks for a great time wherever we met. It's been a nice journey. Hope we meet soon again!

Special thanks to **Matteo**, for being so interested in blobs, and for the great collaboration! Thanks **Stefano**, for also being so interested in blobs. They just couldn't follow our genius ideas. ;)

Thank you all **Mol-toolers** in Uppsala! It was a pity that we moved away, but it was nice to stay connected with you guys. **Tonge**, my dearest partner in particle crime, it was great to start this journey together! Thanks **Linda** and **Liza**, I think we 4 started together. It was great having you, and being lost together in the beginning. ;) **Axel**, who always wanted to work on Luminex with me. Instead we unraveled the compaction-blob-entangling paradoxon together. **Carl-Magnus**, thanks for compacting the hell out of these blobs. And thanks for letting me compact them with you. **Björn**, thanks for fun con-

versations and for always saying funny things. Thanks **Karin**, for speaking Swedish with me even though I must have sounded like a child. **Rasel**, for being a good friend, colleague and retreat roommate. **Caroline**, for working together in the start. I still like the HIV project idea! **Camilla**, for the aptamer-blob collaboration. It was exciting, although we never figured out what it was good for ;). Thanks **Rachel**, **Agata**, **Maria**, **Gucci**, **Junhong**, **Spyros**, **Di**, **Johan V.**, **Elin F.**, **Lotte**, **Elin E.**, **Caroline**, **Johan O.**, **Andries**, **Lei**, **Christina**, **Anne-Li**, **Johanna**, **Joakim**, **Gaelle**, **Felipe** and **Johan B.** You all made this group a nice place to work and hang out. **David**, thanks for all the conversations about god and the world (a literally translated german phrase). **Monica** and **Annika** for being my microfluidic mamas. **Elin E.** for being so endlessly helpful and always having a smile. **Lotte** and **Elin F.** for patiently answering stupid questions and help with selector probes. **Lucy**, for help with samples.

Thanks to the biosensors group in Leuven, Belgium where I spent part of my PhD. Thanks **Jeroen**, **Dragana** and **Federica**, for having me and for being so enthusiastic about RCA. Special thanks to **Daan**, **Kris** and **Karel**. It's been great working with you great scientists! **Elena**, **Iulia**, **Phalguni**, **Deborah** and **Bram** for making me feel so welcome! **Deborah** for making chips (both microfluidic and french fried).

Thanks to the people at Dynal Thermo Fisher in Oslo for being so welcoming and teaching me some particle chemistry. Thanks for the wonderful Christmas party. Special thanks to **Daren** and **Marie**. You really made me feel welcome, even if it was just for one month.

Thanks to the neuroscience bunch in Uppsala that I spent my first months in Uppsala with. Thanks **Johan**, **Lina**, **Klas**, **Martin**, **Anders** and **Kali** for working with you. Thanks to the dudes **Martin** and **Kali**, to **Ida**, **Ninnie**, **Pavol** and **Sanja** for good times, beachvolley, BBQs & indian cooking sessions.

Thanks to the **alfa 4** and **gamma 4** people in Scilifelab, Stockholm for making this floor the best one. **Tim**, for football matches, played and watched, and for beers, brewed and drunk.

Thanks to **Prem**, **Harisha**, and **Aman** for helping me with microfluidic fabrication and discussing bonding problems. And thanks for letting me use your "clean" room!

The Scilifelab **Pub crew**, it's great running the pub with you!

Thanks to the **Nov2k** bunch **Ahmed**, **Kristina**, **Matheus**, **Veronica**, **Elin** and **Annika** for making this happen together. It was hard, but such a creative time and came out so awesome! Thanks to everyone else who was involved and helped out. Special thanks to **Karolina** for great support. To **Bettina** for being so nerd-affine and for your endless creativity. You made it happen.

A special thanks goes to my mentor **Nina**. I really enjoyed our meetings! Thanks for all your help, suggestions and guidance!

Thanks to **Tobias Nilsson** and **Christoph von Ballmoos** at DBB, Stockholm university, for teaching me and letting me use the nanopore instrument.

Thanks to **Single Technologies** for the very exciting collaboration! **Johan**, for your enthusiasm and **Annika** for making things happen!

Thanks to **Qingshan** and the Ozcan group at UCLA for the very exciting mobile phone-RCA collaboration.

To the **Moldia** group: You are the ones I probably spent most of my time with during the last years and you made it a great time! You are amazing colleagues, collaborators and friends. Where to start? **Ivan**, thanks for everything. We worked together on almost all of my last projects. Because we share the same ideas and often have the same thought. Also outside work. You are a true enrichment for me. **Elin L.**, for being such a good friend, for bringing structure into the lab and for having the same great taste in music ;). Thanks **Marco**, for great science, good times outside the lab and for your car. It must have adopted your pessimism. It's always -27 °C cold and very late. **Rongqin**, for teaching me all your RCA skills. **Anja**, for being a great first german connection in the lab, for the nice trips we did together, for being a good friend and collaborator. Our projects were major successes ;). At least we had a good time! **Annika A.**, it was great working with you, especially in the beginning you taught me a lot about microfluidics, for being such a caring person and good friend! **Anna E.**, for all the discussions about work and life and for being a good friend inside and outside the lab. **Tom**, for being a great Uppsala flat-mate, friend and colleague, for always getting shit done. **Magda**, thank you as well for a great time in Uppsala! **Thomas**, for fun times in- and outside the lab. Great to make awesome discoveries with you, for example the antiblobs. **Xiaoyan**, thanks for being such a huge enrichment to the lab, for all the help and tips with analysis matters. You are a genius! **Di**, for all the interesting discussions and sharing your genius ideas. **Didrik**, for deep (very deep) philosophical discussions. **Yonatan**, for funny comments, for help with CADs and other engineering skills, such as ventilator disassembling. **Pavan** and **Jessica**, for your kindness and your hospitality, and always smiling or laughing a lot. ☺ Thanks **Tagrid**, **Amel**, **Sibel**, **Chenglin**, **Nara**, **Mustapha**, **Sara**, **Kilian**, **Giulia**, **J.P.**, **Emma** and all the other wonderful people (I hope I didn't forget anyone. If I did, I am sorry! You know I love you anyways ☺) for creating this great working environment.

Special thanks to my students, and later, assistants. Without you this would really not have been possible! Thanks **Tagrid**, **Sharif**, and **Eva**! **Sharif**, your admiration and enthusiasm for science is endless. Thanks for heated discussions and your (sometimes a bit crazy) ideas. I hope you will invent something amazing. Thanks for all the hard work you put into the blob enrichment project. **Eva**, no words. You saved my butt. Thank you for all your hard work for me and the mobile phone project. And for being a great friend and support!

Thanks **Tim**, **Nara** and **Annika** (my Annika) for proof-reading and sentence-de-germanization. I think you could have been harder on me. Some sentences are still in good old Kant style. ;)

Big thanks to my friends outside work, which I got to spend a bit less time with in the last year. Sorry! Thanks so much for being there, supporting me and making me feel like home in Sweden! **Stefan**, you always follow me around ;). I'm happy to always have one of my best childhood friends close and know you are always there. Thanks **Pieter**, one of my first friends I made in Uppsala. **Pieter** and **Davide**, thanks for the great time when we lived together in Uppsala, for fantastic inter-disciplinary scientific discussions and inter-cultural cooking and party experiences. **Hermany**, it's hard to put it in words. Thanks for being in my life, for being like a brother, and for sharing a wonderful home. **Markus**, also one of the first friends in Sweden and still one of the best, although you prefer spending half a year in a warmer place. ;) I hope we will work together soon. **Simon-Pierre** and **Anna**, you two became like international-swedish family. Thanks for all the brewing, massive Racelette massacres, skiing trips and icehockey matches! **Sim**, thanks for introducing me to hockey and brewing. I hope we win the Swedish homebrew-contest! **Matheus**, thanks for mutual inspirations during and after Nov2k, for wanting to change the world and for being a great friend. **German**, for Columbian hockey-skillz. **Jenny**, for coming to Sweden and making Ivan happy ☺. Thanks **Ninnie** and **Carly** for being great friends!

Hannah, thanks for everything you taught me and for helping me arrive in Uppsala. Thank you for everything!

Thanks to ***Elin and the intergalactic testicles!*** it has been great fun playing with you!

Thanks to my family and friends in Germany. I know you always believe in me! Thank you for that! **Philip**, **Basti**, **Julian**, **Moritz**, **Stefan**, schön mit euch gross zu werden. Das wird uns immer verbinden. Danke **Sören** für die Studizeit. **Maxi**, **Mauri** und **Nora**, meine WG-Familie. Danke!

Mama, ich weiss es war nicht leicht für dich mich nach Schweden gehen zu lassen. Danke für deine niemals endende Unterstützung! **Jana**, danke dass du immer da bist. Wir sind so dicke, man könnte meinen wir sind Geschwister. ☺ **Fees**, Rock'n'roll hat uns zusammen geführt. Du rockst gewaltig! Danke für die Unterstützung und dass du alles immer ein bisschen lustiger gestaltest als es sonst wäre. **Freddy**, danke für die Ablenkung während des Schreibens dieser Arbeit. Frage mich ob da noch Tippfehler von dir drin sind. Danke **Papa**, auch für deine niemals endende Unterstützung und dass du immer an mich glaubst! Danke **Petra**, dass auch du da bist für mich! Danke für die Oxfordisierung meines abstracts. ;) **Oma**, du bist einfach ne Wucht! Danke für deine Liebe und Güte! Du warst immer und bist wie eine zweite Mama für mich. Danke für das öftere Leben-retten und die gute **Erdbeermarmelade**.

Annika, tack för allt! jag kan inte tillräckligt beskriva det i ord hur tacksamt jag är. Du är alltid där för mig. Och det var säkert inte lätt i dem sista veckorna. Tack för du gör livet roligt. Du är den varmhjärtaste person jag känner.

References

1. Morris KV, Mattick JS. The rise of regulatory RNA. *Nature reviews Genetics*. 2014;15(6):423-37. Epub 2014/04/30.
2. Sanger F, Nicklen S, Coulson AR. DNA sequencing with chain-terminating inhibitors. *Proceedings of the National Academy of Sciences of the United States of America*. 1977;74(12):5463-7. Epub 1977/12/01.
3. Maxam AM, Gilbert W. A new method for sequencing DNA. *Proceedings of the National Academy of Sciences of the United States of America*. 1977;74(2):560-4. Epub 1977/02/01.
4. Venter JC, Adams MD, Myers EW, Li PW, Mural RJ, Sutton GG, et al. The sequence of the human genome. *Science*. 2001;291(5507):1304-51. Epub 2001/02/22.
5. Lander ES, Linton LM, Birren B, Nusbaum C, Zody MC, Baldwin J, et al. Initial sequencing and analysis of the human genome. *Nature*. 2001;409(6822):860-921. Epub 2001/03/10.
6. Shendure J, Ji H. Next-generation DNA sequencing. *Nature biotechnology*. 2008;26(10):1135-45. Epub 2008/10/11.
7. Mardis ER. Next-generation DNA sequencing methods. *Annual review of genomics and human genetics*. 2008;9:387-402. Epub 2008/06/26.
8. Shendure J, Lieberman Aiden E. The expanding scope of DNA sequencing. *Nature biotechnology*. 2012;30(11):1084-94. Epub 2012/11/10.
9. Morey M, Fernandez-Marmiesse A, Castineiras D, Fraga JM, Couce ML, Cocho JA. A glimpse into past, present, and future DNA sequencing. *Molecular genetics and metabolism*. 2013;110(1-2):3-24. Epub 2013/06/08.
10. Margulies M, Egholm M, Altman WE, Attiya S, Bader JS, Bemben LA, et al. Genome sequencing in microfabricated high-density picolitre reactors. *Nature*. 2005;437(7057):376-80. Epub 2005/08/02.
11. Ronaghi M, Uhlen M, Nyren P. A sequencing method based on real-time pyrophosphate. *Science*. 1998;281(5375):363, 5. Epub 1998/08/15.
12. Bains W, Smith GC. A novel method for nucleic acid sequence determination. *Journal of theoretical biology*. 1988;135(3):303-7. Epub 1988/12/07.
13. Strezoska Z, Paunesku T, Radosavljevic D, Labat I, Drmanac R, Crkvenjakov R. DNA sequencing by hybridization: 100 bases read by a non-gel-based method. *Proceedings of the National Academy of Sciences of the United States of America*. 1991;88(22):10089-93. Epub 1991/11/15.
14. Shendure J, Porreca GJ, Reppas NB, Lin X, McCutcheon JP, Rosenbaum AM, et al. Accurate multiplex polony sequencing of an evolved bacterial genome. *Science*. 2005;309(5741):1728-32. Epub 2005/08/06.
15. Pihlak A, Bauren G, Hersoug E, Lonnerberg P, Metsis A, Linnarsson S. Rapid genome sequencing with short universal tiling probes. *Nature biotechnology*. 2008;26(6):676-84. Epub 2008/05/27.

16. Drmanac R, Sparks AB, Callow MJ, Halpern AL, Burns NL, Kermani BG, et al. Human genome sequencing using unchained base reads on self-assembling DNA nanoarrays. *Science*. 2010;327(5961):78-81. Epub 2009/11/07.
17. Kasianowicz JJ, Brandin E, Branton D, Deamer DW. Characterization of individual polynucleotide molecules using a membrane channel. *Proceedings of the National Academy of Sciences of the United States of America*. 1996;93(24):13770-3. Epub 1996/11/26.
18. Deamer DW, Akeson M. Nanopores and nucleic acids: prospects for ultrarapid sequencing. *Trends in biotechnology*. 2000;18(4):147-51. Epub 2000/03/31.
19. Howorka S, Cheley S, Bayley H. Sequence-specific detection of individual DNA strands using engineered nanopores. *Nature biotechnology*. 2001;19(7):636-9. Epub 2001/07/04.
20. Levene MJ, Korlach J, Turner SW, Foquet M, Craighead HG, Webb WW. Zero-mode waveguides for single-molecule analysis at high concentrations. *Science*. 2003;299(5607):682-6. Epub 2003/02/01.
21. Ke R, Mignardi M, Pacureanu A, Svedlund J, Botling J, Wahlby C, et al. In situ sequencing for RNA analysis in preserved tissue and cells. *Nature methods*. 2013;10(9):857-60. Epub 2013/07/16.
22. Lee JH, Daugharthy ER, Scheiman J, Kalhor R, Ferrante TC, Terry R, et al. Fluorescent in situ sequencing (FISSEQ) of RNA for gene expression profiling in intact cells and tissues. *Nature protocols*. 2015;10(3):442-58. Epub 2015/02/13.
23. Southern EM. Detection of specific sequences among DNA fragments separated by gel electrophoresis. *Journal of molecular biology*. 1975;98(3):503-17. Epub 1975/11/05.
24. Alwine JC, Kemp DJ, Stark GR. Method for detection of specific RNAs in agarose gels by transfer to diazobenzyloxymethyl-paper and hybridization with DNA probes. *Proceedings of the National Academy of Sciences of the United States of America*. 1977;74(12):5350-4. Epub 1977/12/01.
25. Fodor SP, Read JL, Pirrung MC, Stryer L, Lu AT, Solas D. Light-directed, spatially addressable parallel chemical synthesis. *Science*. 1991;251(4995):767-73. Epub 1991/02/15.
26. Schena M, Shalon D, Davis RW, Brown PO. Quantitative monitoring of gene expression patterns with a complementary DNA microarray. *Science*. 1995;270(5235):467-70. Epub 1995/10/20.
27. Southern EM, Maskos U, Elder JK. Analyzing and comparing nucleic acid sequences by hybridization to arrays of oligonucleotides: evaluation using experimental models. *Genomics*. 1992;13(4):1008-17. Epub 1992/08/01.
28. Gall JG, Pardue ML. Formation and detection of RNA-DNA hybrid molecules in cytological preparations. *Proceedings of the National Academy of Sciences of the United States of America*. 1969;63(2):378-83. Epub 1969/06/01.
29. Jin L, Lloyd RV. In situ hybridization: methods and applications. *Journal of clinical laboratory analysis*. 1997;11(1):2-9. Epub 1997/01/01.
30. Bauman JG, Wiegant J, Borst P, van Duijn P. A new method for fluorescence microscopical localization of specific DNA sequences by in situ hybridization of fluorochromelabelled RNA. *Experimental cell research*. 1980;128(2):485-90. Epub 1980/08/01.
31. Landegren U, Kaiser R, Sanders J, Hood L. A ligase-mediated gene detection technique. *Science*. 1988;241(4869):1077-80. Epub 1988/08/26.
32. Wu DY, Wallace RB. The ligation amplification reaction (LAR)--amplification of specific DNA sequences using sequential rounds of template-dependent ligation. *Genomics*. 1989;4(4):560-9. Epub 1989/05/01.

33. Barany F. Genetic disease detection and DNA amplification using cloned thermostable ligase. *Proceedings of the National Academy of Sciences of the United States of America*. 1991;88(1):189-93. Epub 1991/01/01.
34. Nilsson M, Malmgren H, Samiotaki M, Kwiatkowski M, Chowdhary BP, Landegren U. Padlock probes: circularizing oligonucleotides for localized DNA detection. *Science*. 1994;265(5181):2085-8. Epub 1994/09/30.
35. Mullis K, Faloona F, Scharf S, Saiki R, Horn G, Erlich H. Specific enzymatic amplification of DNA in vitro: the polymerase chain reaction. *Cold Spring Harbor symposia on quantitative biology*. 1986;51 Pt 1:263-73. Epub 1986/01/01.
36. Saiki RK, Gelfand DH, Stoffel S, Scharf SJ, Higuchi R, Horn GT, et al. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science*. 1988;239(4839):487-91. Epub 1988/01/29.
37. Gibson UE, Heid CA, Williams PM. A novel method for real time quantitative RT-PCR. *Genome research*. 1996;6(10):995-1001. Epub 1996/10/01.
38. Heid CA, Stevens J, Livak KJ, Williams PM. Real time quantitative PCR. *Genome research*. 1996;6(10):986-94. Epub 1996/10/01.
39. Tyagi S, Kramer FR. Molecular beacons: probes that fluoresce upon hybridization. *Nature biotechnology*. 1996;14(3):303-8. Epub 1996/03/01.
40. Holland PM, Abramson RD, Watson R, Gelfand DH. Detection of specific polymerase chain reaction product by utilizing the 5'----3' exonuclease activity of *Thermus aquaticus* DNA polymerase. *Proceedings of the National Academy of Sciences of the United States of America*. 1991;88(16):7276-80. Epub 1991/08/15.
41. Compton J. Nucleic acid sequence-based amplification. *Nature*. 1991;350(6313):91-2. Epub 1991/03/07.
42. Walker GT, Fraiser MS, Schram JL, Little MC, Nadeau JG, Malinowski DP. Strand displacement amplification--an isothermal, in vitro DNA amplification technique. *Nucleic Acids Res*. 1992;20(7):1691-6. Epub 1992/04/11.
43. Walker GT, Little MC, Nadeau JG, Shank DD. Isothermal in vitro amplification of DNA by a restriction enzyme/DNA polymerase system. *Proceedings of the National Academy of Sciences of the United States of America*. 1992;89(1):392-6. Epub 1992/01/01.
44. Notomi T, Okayama H, Masubuchi H, Yonekawa T, Watanabe K, Amino N, et al. Loop-mediated isothermal amplification of DNA. *Nucleic Acids Res*. 2000;28(12):E63. Epub 2000/06/28.
45. Dean FB, Nelson JR, Giesler TL, Lasken RS. Rapid amplification of plasmid and phage DNA using Phi 29 DNA polymerase and multiply-primed rolling circle amplification. *Genome research*. 2001;11(6):1095-9. Epub 2001/05/31.
46. Fire A, Xu SQ. Rolling replication of short DNA circles. *Proceedings of the National Academy of Sciences of the United States of America*. 1995;92(10):4641-5. Epub 1995/05/09.
47. Lizardi PM, Huang X, Zhu Z, Bray-Ward P, Thomas DC, Ward DC. Mutation detection and single-molecule counting using isothermal rolling-circle amplification. *Nat Genet*. 1998;19(3):225-32. Epub 1998/07/14.
48. Dahl F, Baner J, Gullberg M, Mendel-Hartvig M, Landegren U, Nilsson M. Circle-to-circle amplification for precise and sensitive DNA analysis. *Proceedings of the National Academy of Sciences of the United States of America*. 2004;101(13):4548-53. Epub 2004/04/09.

49. Nilsson M, Krejci K, Koch J, Kwiatkowski M, Gustavsson P, Landegren U. Padlock probes reveal single-nucleotide differences, parent of origin and in situ distribution of centromeric sequences in human chromosomes 13 and 21. *Nature genetics*. 1997;16(3):252-5. Epub 1997/07/01.
50. Baner J, Nilsson M, Mendel-Hartvig M, Landegren U. Signal amplification of padlock probes by rolling circle replication. *Nucleic Acids Res*. 1998;26(22):5073-8. Epub 1998/11/04.
51. Hardenbol P, Baner J, Jain M, Nilsson M, Namsaraev EA, Karlin-Neumann GA, et al. Multiplexed genotyping with sequence-tagged molecular inversion probes. *Nature biotechnology*. 2003;21(6):673-8. Epub 2003/05/06.
52. Blondal T, Thorisdottir A, Unnsteinsdottir U, Hjorleifsdottir S, Aevarsson A, Ernstsson S, et al. Isolation and characterization of a thermostable RNA ligase 1 from a *Thermus scotoductus* bacteriophage TS2126 with good single-stranded DNA ligation properties. *Nucleic Acids Res*. 2005;33(1):135-42. Epub 2005/01/12.
53. Lou DI, Hussmann JA, McBee RM, Acevedo A, Andino R, Press WH, et al. High-throughput DNA sequencing errors are reduced by orders of magnitude using circle sequencing. *Proceedings of the National Academy of Sciences of the United States of America*. 2013;110(49):19872-7. Epub 2013/11/19.
54. Stenberg J, Dahl F, Landegren U, Nilsson M. PieceMaker: selection of DNA fragments for selector-guided multiplex amplification. *Nucleic Acids Res*. 2005;33(8):e72. Epub 2005/04/30.
55. Dahl F, Gullberg M, Stenberg J, Landegren U, Nilsson M. Multiplex amplification enabled by selective circularization of large sets of genomic DNA fragments. *Nucleic Acids Res*. 2005;33(8):e71. Epub 2005/04/30.
56. Dahl F, Stenberg J, Fredriksson S, Welch K, Zhang M, Nilsson M, et al. Multigene amplification and massively parallel sequencing for cancer mutation discovery. *Proceedings of the National Academy of Sciences of the United States of America*. 2007;104(22):9387-92. Epub 2007/05/23.
57. Johansson H, Isaksson M, Sorqvist EF, Roos F, Stenberg J, Sjoblom T, et al. Targeted resequencing of candidate genes using selector probes. *Nucleic Acids Res*. 2011;39(2):e8. Epub 2010/11/10.
58. Mathot L, Falk-Sorqvist E, Moens L, Allen M, Sjoblom T, Nilsson M. Automated genotyping of biobank samples by multiplex amplification of insertion/deletion polymorphisms. *PloS one*. 2012;7(12):e52750. Epub 2013/01/10.
59. Moens LN, Falk-Sorqvist E, Ljungstrom V, Mattsson J, Sundstrom M, La Fleur L, et al. HaloPlex Targeted Resequencing for Mutation Detection in Clinical Formalin-Fixed, Paraffin-Embedded Tumor Samples. *The Journal of molecular diagnostics : JMD*. 2015. Epub 2015/09/12.
60. Walt DR. Optical Methods for Single Molecule Detection and Analysis. *Analytical chemistry*. 2012. Epub 2012/12/12.
61. Geiss GK, Bumgarner RE, Birditt B, Dahl T, Dowidar N, Dunaway DL, et al. Direct multiplexed measurement of gene expression with color-coded probe pairs. *Nature biotechnology*. 2008;26(3):317-25. Epub 2008/02/19.
62. Femino AM, Fay FS, Fogarty K, Singer RH. Visualization of single RNA transcripts in situ. *Science*. 1998;280(5363):585-90. Epub 1998/05/09.
63. Raj A, van den Bogaard P, Rifkin SA, van Oudenaarden A, Tyagi S. Imaging individual mRNA molecules using multiple singly labeled probes. *Nature methods*. 2008;5(10):877-9. Epub 2008/09/23.

64. Lubeck E, Coskun AF, Zhiyentayev T, Ahmad M, Cai L. Single-cell in situ RNA profiling by sequential hybridization. *Nature methods*. 2014;11(4):360-1. Epub 2014/04/01.
65. Chen KH, Boettiger AN, Moffitt JR, Wang S, Zhuang X. RNA imaging. Spatially resolved, highly multiplexed RNA profiling in single cells. *Science*. 2015;348(6233):aaa6090. Epub 2015/04/11.
66. Kern D, Collins M, Fultz T, Detmer J, Hamren S, Peterkin JJ, et al. An enhanced-sensitivity branched-DNA assay for quantification of human immunodeficiency virus type 1 RNA in plasma. *J Clin Microbiol*. 1996;34(12):3196-202. Epub 1996/12/01.
67. Player AN, Shen LP, Kenny D, Antao VP, Kolberg JA. Single-copy gene detection using branched DNA (bDNA) in situ hybridization. *The journal of histochemistry and cytochemistry : official journal of the Histochemistry Society*. 2001;49(5):603-12. Epub 2001/04/17.
68. Collins ML, Irvine B, Tyner D, Fine E, Zayati C, Chang C, et al. A branched DNA signal amplification assay for quantification of nucleic acid targets below 100 molecules/ml. *Nucleic Acids Res*. 1997;25(15):2979-84. Epub 1997/08/01.
69. Dirks RM, Pierce NA. Triggered amplification by hybridization chain reaction. *Proceedings of the National Academy of Sciences of the United States of America*. 2004;101(43):15275-8. Epub 2004/10/20.
70. Rissin DM, Kan CW, Campbell TG, Howes SC, Fournier DR, Song L, et al. Single-molecule enzyme-linked immunosorbent assay detects serum proteins at subfemtomolar concentrations. *Nature biotechnology*. 2010;28(6):595-9. Epub 2010/05/25.
71. Gorris HH, Rissin DM, Walt DR. Stochastic inhibitor release and binding from single-enzyme molecules. *Proceedings of the National Academy of Sciences of the United States of America*. 2007;104(45):17680-5. Epub 2007/10/30.
72. Sykes PJ, Neoh SH, Brisco MJ, Hughes E, Condon J, Morley AA. Quantitation of targets for PCR by use of limiting dilution. *BioTechniques*. 1992;13(3):444-9. Epub 1992/09/01.
73. Vogelstein B, Kinzler KW. Digital PCR. *Proceedings of the National Academy of Sciences of the United States of America*. 1999;96(16):9236-41. Epub 1999/08/04.
74. Diehl F, Li M, He Y, Kinzler KW, Vogelstein B, Dressman D. BEAMing: single-molecule PCR on microparticles in water-in-oil emulsions. *Nature methods*. 2006;3(7):551-9. Epub 2006/06/23.
75. Ottesen EA, Hong JW, Quake SR, Leadbetter JR. Microfluidic digital PCR enables multigene analysis of individual environmental bacteria. *Science*. 2006;314(5804):1464-7. Epub 2006/12/02.
76. Thorsen T, Maerkl SJ, Quake SR. Microfluidic large-scale integration. *Science*. 2002;298(5593):580-4. Epub 2002/09/28.
77. Hindson BJ, Ness KD, Masquelier DA, Belgrader P, Heredia NJ, Makarewicz AJ, et al. High-throughput droplet digital PCR system for absolute quantitation of DNA copy number. *Analytical chemistry*. 2011;83(22):8604-10. Epub 2011/11/01.
78. Pekin D, Skhiri Y, Baret JC, Le Corre D, Mazutis L, Salem CB, et al. Quantitative and sensitive detection of rare mutations using droplet-based microfluidics. *Lab on a chip*. 2011;11(13):2156-66. Epub 2011/05/20.

79. Larsson C, Koch J, Nygren A, Janssen G, Raap AK, Landegren U, et al. In situ genotyping individual DNA molecules by target-primed rolling-circle amplification of padlock probes. *Nature methods*. 2004;1(3):227-32. Epub 2005/03/23.
80. Soderberg O, Gullberg M, Jarvius M, Ridderstrale K, Leuchowius KJ, Jarvius J, et al. Direct observation of individual endogenous protein complexes in situ by proximity ligation. *Nature methods*. 2006;3(12):995-1000. Epub 2006/10/31.
81. Blab GA, Schmidt T, Nilsson M. Homogeneous detection of single rolling circle replication products. *Analytical chemistry*. 2004;76(2):495-8. Epub 2004/01/15.
82. Melin J, Johansson H, Soderberg O, Nikolajeff F, Landegren U, Nilsson M, et al. Thermoplastic microfluidic platform for single-molecule detection, cell culture, and actuation. *Analytical chemistry*. 2005;77(22):7122-30. Epub 2005/11/16.
83. Blab GA, Oellerich S, Schumm R, Schmidt T. Simultaneous wide-field imaging and spectroscopy of localized fluorophores. *Optics letters*. 2004;29(7):727-9. Epub 2004/04/10.
84. Jarvius J, Melin J, Goransson J, Stenberg J, Fredriksson S, Gonzalez-Rey C, et al. Digital quantification using amplified single-molecule detection. *Nature methods*. 2006;3(9):725-7. Epub 2006/08/25.
85. Goransson J, Wahlby C, Isaksson M, Howell WM, Jarvius J, Nilsson M. A single molecule array for digital targeted molecular analyses. *Nucleic Acids Res*. 2009;37(1):e7. Epub 2008/11/27.
86. Baner J, Gyarmati P, Yacoub A, Hakhverdyan M, Stenberg J, Ericsson O, et al. Microarray-based molecular detection of foot-and-mouth disease, vesicular stomatitis and swine vesicular disease viruses, using padlock probes. *Journal of virological methods*. 2007;143(2):200-6. Epub 2007/04/25.
87. Eriksson R, Jobs M, Ekstrand C, Ullberg M, Herrmann B, Landegren U, et al. Multiplex and quantifiable detection of nucleic acid from pathogenic fungi using padlock probes, generic real time PCR and specific suspension array readout. *Journal of microbiological methods*. 2009;78(2):195-202. Epub 2009/06/06.
88. Ke RQ, Zorzet A, Goransson J, Lindegren G, Sharifi-Mood B, Chinikar S, et al. Colorimetric Nucleic Acid Testing Assay for RNA Virus Detection Based on Circle-to-Circle Amplification of Padlock Probes. *J Clin Microbiol*. 2011;49(12):4279-85.
89. Goransson J, Ke R, Nong RY, Howell WM, Karman A, Grawe J, et al. Rapid identification of bio-molecules applied for detection of biosecurity agents using rolling circle amplification. *PloS one*. 2012;7(2):e31068. Epub 2012/03/03.
90. Engstrom A, Zardan Gomez de la Torre T, Stromme M, Nilsson M, Herthnek D. Detection of rifampicin resistance in *Mycobacterium tuberculosis* by padlock probes and magnetic nanobead-based readout. *PloS one*. 2013;8(4):e62015. Epub 2013/05/01.
91. Mezger A, Ohrmalm C, Herthnek D, Blomberg J, Nilsson M. Detection of rotavirus using padlock probes and rolling circle amplification. *PloS one*. 2014;9(11):e111874. Epub 2014/11/05.
92. Mezger A, Gullberg E, Goransson J, Zorzet A, Herthnek D, Tano E, et al. A general method for rapid determination of antibiotic susceptibility and species in bacterial infections. *J Clin Microbiol*. 2015;53(2):425-32. Epub 2014/11/21.

93. Mezger A, Fock J, Antunes P, Osterberg FW, Boisen A, Nilsson M, et al. Scalable DNA-Based Magnetic Nanoparticle Agglutination Assay for Bacterial Detection in Patient Samples. *ACS nano*. 2015;9(7):7374-82. Epub 2015/07/15.
94. Larsson C, Grundberg I, Soderberg O, Nilsson M. In situ detection and genotyping of individual mRNA molecules. *Nature methods*. 2010;7(5):395-7. Epub 2010/04/13.
95. Grundberg I, Kiflemariam S, Mignardi M, Imgenberg-Kreuz J, Edlund K, Micke P, et al. In situ mutation detection and visualization of intratumor heterogeneity for cancer research and diagnostics. *Oncotarget*. 2013;4(12):2407-18. Epub 2013/11/28.
96. Mignardi M, Mezger A, Qian X, La Fleur L, Botling J, Larsson C, et al. Oligonucleotide gap-fill ligation for mutation detection and sequencing in situ. *Nucleic Acids Res*. 2015;43(22):e151. Epub 2015/08/05.
97. Weibrecht I, Lundin E, Kiflemariam S, Mignardi M, Grundberg I, Larsson C, et al. In situ detection of individual mRNA molecules and protein complexes or post-translational modifications using padlock probes combined with the in situ proximity ligation assay. *Nature protocols*. 2013;8(2):355-72. Epub 2013/01/26.
98. Weibrecht I, Gavrilovic M, Lindbom L, Landegren U, Wahlby C, Soderberg O. Visualising individual sequence-specific protein-DNA interactions in situ. *New biotechnology*. 2012;29(5):589-98. Epub 2011/09/13.
99. Nilsson M, Gullberg M, Dahl F, Szuhai K, Raap AK. Real-time monitoring of rolling-circle amplification using a modified molecular beacon design. *Nucleic Acids Res*. 2002;30(14):e66. Epub 2002/07/24.
100. Cheglakov Z, Weizmann Y, Braunschweig AB, Wilner OI, Willner I. Increasing the complexity of periodic protein nanostructures by the rolling-circle-amplified synthesis of aptamers. *Angew Chem Int Ed Engl*. 2008;47(1):126-30. Epub 2007/11/27.
101. Claussan CM, Arngarden L, Ishaq O, Klaesson A, Kuhnemund M, Grannas K, et al. Compaction of rolling circle amplification products increases signal integrity and signal-to-noise ratio. *Scientific reports*. 2015;5:12317. Epub 2015/07/24.
102. Cheng W, Yan F, Ding L, Ju H, Yin Y. Cascade signal amplification strategy for subattomolar protein detection by rolling circle amplification and quantum dots tagging. *Analytical chemistry*. 2010;82(8):3337-42. Epub 2010/03/30.
103. Stromberg M, Goransson J, Gunnarsson K, Nilsson M, Svedlindh P, Stromme M. Sensitive molecular diagnostics using volume-amplified magnetic nanobeads. *Nano letters*. 2008;8(3):816-21. Epub 2008/02/06.
104. Stromberg M, Zardan Gomez de la Torre T, Goransson J, Gunnarsson K, Nilsson M, Svedlindh P, et al. Multiplex detection of DNA sequences using the volume-amplified magnetic nanobead detection assay. *Analytical chemistry*. 2009;81(9):3398-406. Epub 2009/04/02.
105. Beyer S, Nickels P, Simmel FC. Periodic DNA nanotemplates synthesized by rolling circle amplification. *Nano letters*. 2005;5(4):719-22. Epub 2005/04/14.
106. Hao R, Xing R, Xu Z, Hou Y, Gao S, Sun S. Synthesis, functionalization, and biomedical applications of multifunctional magnetic nanoparticles. *Adv Mater*. 2010;22(25):2729-42. Epub 2010/05/18.
107. Gijs MA, Lacharme F, Lehmann U. Microfluidic applications of magnetic particles for biological analysis and catalysis. *Chemical reviews*. 2010;110(3):1518-63. Epub 2009/12/08.

108. Manz A, Graber N, Widmer HM. Miniaturized Total Chemical-Analysis Systems - a Novel Concept for Chemical Sensing. *Sensor Actuat B-Chem.* 1990;1(1-6):244-8.
109. Manz A, Verpoorte E, Raymond DE, Effenhauser CS, Burggraf N, Widmer HM. μ -TAS - MINIATURIZED TOTAL CHEMICAL ANALYSIS SYSTEMS. *Mesa Mg.* 1995:5-27.
110. Whitesides GM. The origins and the future of microfluidics. *Nature.* 2006;442(7101):368-73. Epub 2006/07/28.
111. Yager P, Edwards T, Fu E, Helton K, Nelson K, Tam MR, et al. Microfluidic diagnostic technologies for global public health. *Nature.* 2006;442(7101):412-8. Epub 2006/07/28.
112. Darmanis S, Gallant CJ, Marinescu VD, Niklasson M, Segerman A, Flamourakis G, et al. Simultaneous Multiplexed Measurement of RNA and Proteins in Single Cells. *Cell reports.* 2016;14(2):380-9. Epub 2016/01/11.
113. Zeisel A, Munoz-Manchado AB, Codeluppi S, Lonnerberg P, La Manno G, Jureus A, et al. Brain structure. Cell types in the mouse cortex and hippocampus revealed by single-cell RNA-seq. *Science.* 2015;347(6226):1138-42. Epub 2015/02/24.
114. Duffy DC, McDonald JC, Schueller OJ, Whitesides GM. Rapid Prototyping of Microfluidic Systems in Poly(dimethylsiloxane). *Analytical chemistry.* 1998;70(23):4974-84. Epub 1998/12/01.
115. Kopp MU, Mello AJ, Manz A. Chemical amplification: continuous-flow PCR on a chip. *Science.* 1998;280(5366):1046-8. Epub 1998/06/06.
116. Folch A, Ayon A, Hurtado O, Schmidt MA, Toner M. Molding of deep polydimethylsiloxane microstructures for microfluidics and biological applications. *Journal of biomechanical engineering.* 1999;121(1):28-34. Epub 1999/03/18.
117. de Jong J, Lammertink RG, Wessling M. Membranes and microfluidics: a review. *Lab on a chip.* 2006;6(9):1125-39. Epub 2006/08/25.
118. Gan W, Zhuang B, Zhang P, Han J, Li CX, Liu P. A filter paper-based microdevice for low-cost, rapid, and automated DNA extraction and amplification from diverse sample types. *Lab on a chip.* 2014;14(19):3719-28. Epub 2014/07/30.
119. Pollack MG, Fair RB, Shenderov AD. Electrowetting-based actuation of liquid droplets for microfluidic applications. *Applied Physics Letters.* 2000;77(11):1725-6.
120. Pollack MG, Shenderov AD, Fair RB. Electrowetting-based actuation of droplets for integrated microfluidics. *Lab on a chip.* 2002;2(2):96-101. Epub 2004/04/22.
121. Wheeler AR. Chemistry. Putting electrowetting to work. *Science.* 2008;322(5901):539-40. Epub 2008/10/25.
122. Vergauwe N, Witters D, Ceyssens F, Vermeir S, Verbruggen B, Puers R, et al. A versatile electrowetting-based digital microfluidic platform for quantitative homogeneous and heterogeneous bio-assays. *J Micromech Microeng.* 2011;21(5).
123. Ng AH, Choi K, Luoma RP, Robinson JM, Wheeler AR. Digital microfluidic magnetic separation for particle-based immunoassays. *Analytical chemistry.* 2012;84(20):8805-12. Epub 2012/09/28.
124. Choi K, Ng AH, Fobel R, Wheeler AR. Digital microfluidics. *Annu Rev Anal Chem (Palo Alto Calif).* 2012;5:413-40. Epub 2012/04/25.

125. Witters D, Vergauwe N, Vermeir S, Ceyssens F, Liekens S, Puers R, et al. Biofunctionalization of electrowetting-on-dielectric digital microfluidic chips for miniaturized cell-based applications. *Lab on a chip*. 2011;11(16):2790-4. Epub 2011/07/02.
126. Baviere R, Boutet J, Fouillet Y. Dynamics of droplet transport induced by electrowetting actuation. *Microfluid Nanofluid*. 2008;4(4):287-94.
127. Roux JM, Achard JL, Fouillet Y. Forces and charges on an undeformable droplet in the DC field of a plate condenser. *J Electrostat*. 2008;66(5-6):283-93.
128. Fouillet Y, Achard JL. Digital microfluidic and biotechnology. *Cr Phys*. 2004;5(5):577-88.
129. Fouillet Y, Jary D, Chabrol C, Claustre P, Peponnet C. Digital microfluidic design and optimization of classic and new fluidic functions for lab on a chip systems. *Microfluid Nanofluid*. 2008;4(3):159-65.
130. Melin J, Jarvius J, Goransson J, Nilsson M. Homogeneous amplified single-molecule detection: Characterization of key parameters. *Analytical biochemistry*. 2007;368(2):230-8. Epub 2007/06/19.
131. Tanaka Y, Xi H, Sato K, Mawatari K, Renberg B, Nilsson M, et al. Single-molecule DNA patterning and detection by padlock probing and rolling circle amplification in microchannels for analysis of small sample volumes. *Analytical chemistry*. 2011;83(9):3352-7. Epub 2011/04/06.
132. Sato K, Tachihara A, Renberg B, Mawatari K, Tanaka Y, Jarvius J, et al. Microbead-based rolling circle amplification in a microchip for sensitive DNA detection. *Lab on a chip*. 2010;10(10):1262-6. Epub 2010/05/07.
133. Ozcan A. Mobile phones democratize and cultivate next-generation imaging, diagnostics and measurement tools. *Lab on a chip*. 2014;14(17):3187-94. Epub 2014/03/22.
134. Coulter HW. Means for counting particles suspended in a fluid. Google Patents; 1953.
135. Ito T, Sun L, Crooks RM. Simultaneous determination of the size and surface charge of individual nanoparticles using a carbon nanotube-based Coulter counter. *Analytical chemistry*. 2003;75(10):2399-406. Epub 2003/08/16.
136. Roberts GS, Kozak D, Anderson W, Broom MF, Vogel R, Trau M. Tunable nano/micropores for particle detection and discrimination: scanning ion occlusion spectroscopy. *Small*. 2010;6(23):2653-8. Epub 2010/10/28.
137. Yang AK, Lu H, Wu SY, Kwok HC, Ho HP, Yu S, et al. Detection of Pantone-Valentine Leukocidin DNA from methicillin-resistant *Staphylococcus aureus* by resistive pulse sensing and loop-mediated isothermal amplification with gold nanoparticles. *Analytica chimica acta*. 2013;782:46-53. Epub 2013/05/28.
138. Smeets RM, Kowalczyk SW, Hall AR, Dekker NH, Dekker C. Translocation of RecA-coated double-stranded DNA through solid-state nanopores. *Nano letters*. 2009;9(9):3089-96. Epub 2008/12/05.
139. Henry JP, Chich JF, Goldschmidt D, Thieffry M. Blockade of a mitochondrial cationic channel by an addressing peptide: an electrophysiological study. *The Journal of membrane biology*. 1989;112(2):139-47. Epub 1989/12/01.
140. Saharil F, Ahlford, A., Kühnemund, M., Skolimowski, M., Conde, A., Dufva, M., Nilsson, M., Brivio, M., van der Wijngaart, W., Haraldsson, T. . Ligation-based mutation detection and RCA in surface un-modified OSTe+ polymer microfluidic chambers. *Transducers & Euroensors 2013 ed2013*. p. 357-60.
141. Kuhnemund M, Witters D, Nilsson M, Lammertyn J. Circle-to-circle amplification on a digital microfluidic chip for amplified single molecule detection. *Lab on a chip*. 2014;14(16):2983-92. Epub 2014/06/18.

142. Sato K, Ishii R, Sasaki N, Nilsson M. Bead-based padlock rolling circle amplification for single DNA molecule counting. *Analytical biochemistry*. 2013. Epub 2013/03/08.
143. Kuhnemund M, Nilsson M. Digital quantification of rolling circle amplified single DNA molecules in a resistive pulse sensing nanopore. *Biosensors & bioelectronics*. 2015;67:11-7. Epub 2014/07/09.
144. Li J, Deng T, Chu X, Yang R, Jiang J, Shen G, et al. Rolling circle amplification combined with gold nanoparticle aggregates for highly sensitive identification of single-nucleotide polymorphisms. *Analytical chemistry*. 2010;82(7):2811-6. Epub 2010/03/03.
145. Zardan Gomez de la Torre T, Stromberg M, Russell C, Goransson J, Nilsson M, Svedlindh P, et al. Investigation of immobilization of functionalized magnetic nanobeads in rolling circle amplified DNA coils. *The journal of physical chemistry B*. 2010;114(10):3707-13. Epub 2010/02/24.
146. Russell C, Welch K, Jarvius J, Cai Y, Brucas R, Nikolajeff F, et al. Gold nanowire based electrical DNA detection using rolling circle amplification. *ACS nano*. 2014;8(2):1147-53. Epub 2014/01/18.
147. Russell C, Roy S, Ganguly S, Qian X, Caruthers MH, Nilsson M. Formation of Silver Nanostructures by Rolling Circle Amplification Using Boranephosphonate-Modified Nucleotides. *Analytical chemistry*. 2015;87(13):6660-6. Epub 2015/06/11.
148. Mahmoudian L, Kaji N, Tokeshi M, Nilsson M, Baba Y. Rolling circle amplification and circle-to-circle amplification of a specific gene integrated with electrophoretic analysis on a single chip. *Analytical chemistry*. 2008;80(7):2483-90. Epub 2008/03/01.
149. Mahmoudian L, Melin J, Mohamadi MR, Yamada K, Ohta M, Kaji N, et al. Microchip electrophoresis for specific gene detection of the pathogenic bacteria *V. cholerae* by circle-to-circle amplification. *Analytical sciences : the international journal of the Japan Society for Analytical Chemistry*. 2008;24(3):327-32. Epub 2008/03/12.
150. Schmidt TL, Beliveau BJ, Uca YO, Theilmann M, Da Cruz F, Wu CT, et al. Scalable amplification of strand subsets from chip-synthesized oligonucleotide libraries. *Nature communications*. 2015;6:8634. Epub 2015/11/17.

Acta Universitatis Upsaliensis

*Digital Comprehensive Summaries of Uppsala Dissertations
from the Faculty of Medicine 1189*

Editor: The Dean of the Faculty of Medicine

A doctoral dissertation from the Faculty of Medicine, Uppsala University, is usually a summary of a number of papers. A few copies of the complete dissertation are kept at major Swedish research libraries, while the summary alone is distributed internationally through the series Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine. (Prior to January, 2005, the series was published under the title "Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine".)

Distribution: publications.uu.se
urn:nbn:se:uu:diva-279372



ACTA
UNIVERSITATIS
UPSALIENSIS
UPPSALA
2016