HANDS-FREE CYTOMETRY OF WHOLE BLOOD

*Controlled antibody release from hydrogels for
on-chip cell staining*

Xichen Zhang

HANDS-FREE CYTOMETRY OF WHOLE BLOOD

*Controlled antibody release from hydrogels for
on-chip cell staining*

Xichen Zhang
University of Twente
January 2017

Members of the graduation committee:

Chairman

Prof. dr. ir. J. W. M. Hilgenkamp University of Twente

Promotor

Prof. dr. L. W. M. M. Terstappen University of Twente

Co-promotor

Dr. M. Beck University of Twente

Members

Prof. dr. D. W. Grijpma University of Twente

Prof. dr. ir. J. F. Dijksman University of Twente

Prof. dr. J. F. C. Glatz University of Maastricht

Prof. dr. E. M. J. Verpoorte University of Groningen

Referee

Dr. J. Slomp Medisch Spectrum Twente

The research reported in this thesis was carried out at the Medical Cell Biophysics group within the Faculty of Science and Technology, and the MIRA Institute at University of Twente, Enschede, the Netherlands. This work was part of the “InstantCount” project which is financially supported by ERC starting grant, No. 282276.

Copyright © Xichen Zhang, Enschede, the Netherlands

All rights reserved. No part of this work may be reproduced by print, photocopy, or any other means without written permission of the author.

ISBN:	978-90-365-4274-6
DOI:	10.3990/1.9789036542746
Cover Image:	SCIMAGE.CN
Cover Design:	Xichen Zhang
Publisher:	Gildeprint, Enschede, the Netherlands

HANDS-FREE CYTOMETRY OF WHOLE BLOOD

*Controlled antibody release from hydrogels for
on-chip cell staining*

Dissertation

to obtain
the degree of doctor at the University of Twente,
on the authority of the rector magnificus,
prof. dr. T. T. M. Palstra,
on account of the decision of the graduation committee,
to be publicly defended
on Wednesday 1 February 2017 at 14:45 hrs

by
Xichen Zhang
born on 25 April 1987
in Wuhan, China

This dissertation has been approved by:

Prof. dr. L. W. M. M. Terstappen (promotor)

Dr. M. Beck (co-promotor)

Table of Contents

Introduction	1
Aim of the thesis.....	2
Thesis outline	3
1. On-chip sample preparation for point-of-care cellular analysis of blood	7
Abstract.....	8
1.1 Introduction.....	9
1.2 Reagent-free cell separation based on physical characteristics	11
1.2.1 Filtration.....	11
1.2.2 Deterministic lateral displacement.....	15
1.2.3 Pinched flow fractionation/Hydrodynamic filtration.....	18
1.2.4 Inertial microfluidics.....	19
1.2.5 Dielectrophoresis.....	22
1.2.6 Lab-on-a-disk.....	24
1.3 Biomarker mediated cell identification and sorting based on immune characteristics	25
1.3.1 On-chip immunostaining and cell identification	25
1.3.2 On-chip immunomagnetic cell separation.....	28
1.3.3 On-chip immunocapture.....	30
1.4 Outlook	33
References.....	34
2. Real-time <i>in situ</i> fluorescence imaging system for the measurement of on-chip antibody release	39
Abstract.....	40
2.1 Introduction.....	41
2.2 Flow chamber fabrication and layer preparation	43
2.3 Fluorescence imaging system.....	45
2.4 Characterization of layer thickness.....	46
2.5 Characterization of chamber height.....	47
2.6 Correction of measured fluorescence intensity	48

2.7 Calibration of fluorescence intensity against the quantity of APC- α CD3.....	49
2.8 Measurement of release kinetics	50
2.9 Conclusion	53
References.....	54
3. Controlled antibody release from gelatin for on-chip sample preparation.....	55
Abstract.....	56
3.1 Introduction.....	57
3.2 Experiments.....	59
3.2.1 Flow chamber and cell counting chamber fabrication	59
3.2.2 Layer preparation.....	61
3.2.3 Released molecules.....	61
3.2.4 Fluorescence imaging	61
3.2.5 Layer characterization.....	62
3.2.6 Measurement of equilibrium mass swelling ratios.....	63
3.2.7 Measurement of release kinetics in flow chambers.....	64
3.2.8 Measurement of antibody distribution and intensity of stained cells in counting chambers.....	64
3.3 Results and discussion	65
3.3.1 Diffusion-controlled release mechanism and the influence of layer thickness on the release.....	65
3.3.2 Influence of layer preparation conditions on the release and possible mechanism.....	67
3.3.3 Influence of reagent size on the release.....	70
3.3.4 Influence of electrostatic interactions on the release	71
3.3.5 Implication of antibody release kinetics for on-chip cell staining.....	72
3.4 Conclusion	75
3.5 Supporting information.....	76
3.5.1 Reproducibility of the gelatin layers prepared using “standard conditions” and chamber heights	76
3.5.2 Exclusion of perfusion in the release process	77
3.5.3 Statistical analysis of different release kinetics.....	78
3.5.4 FT-IR spectroscopy	79

References.....	81
4. Temperature switch cytometry – releasing antibody on demand from inkjet-printed gelatin for on-chip immunostaining.....	83
Abstract.....	84
4.1 Introduction.....	85
4.2 Experiments.....	86
4.2.1 Flow chamber and cell counting chamber fabrication	86
4.2.2 Inkjet printing and layer maturation	87
4.2.3 Layer characterization.....	88
4.2.4 Measurement of release kinetics in flow chambers.....	89
4.2.5 Measurement of antibody distribution and intensity of stained cells in cell counting chambers.....	90
4.3 Results and discussion.....	91
4.3.1 Delayed antibody release induced by layer maturation	91
4.3.2 Temperature switched antibody release with on demand antibody-sample mixing	93
4.3.3 Optimized on-chip immunostaining.....	95
4.4 Conclusion	97
4.5 Supporting information.....	99
4.5.1 Development of release kinetics of APC- α CD3 and its stability under different maturation conditions.....	99
4.5.2 Temperature dependent layer integrity	99
4.5.3 Distribution of stained cells in cell counting chambers	100
4.5.4 Comparison of release kinetics between APC- α CD3 and PerCP- α CD4 from different printed layers	100
References.....	101
5. Automated fabrication of microfluidic CD4-counting chips containing staining agents using drop-on-demand printing methods	103
Abstract.....	104
5.1 Introduction.....	105
5.2 Experiments.....	108
5.2.1 Materials	108

5.2.2 Equipment	109
5.2.3 Chamber fabrication by piezo-actuated deposition.....	110
5.2.4 Gelatin/antibody deposition by inkjet printing.....	110
5.2.5 Leukocyte depletion and leukocyte spiking.....	111
5.3 Results and discussion	111
5.3.1 Fabrication of counting chambers by printing.....	111
5.3.2 Inkjet printing of hydrogel/reagent layers.....	114
5.3.3 Optimization of reagent release from printed hydrogel layers	119
5.3.4 Performance of fully printed CD4 counting chambers	124
5.4 Conclusion	128
5.5 Supporting Information.....	130
References.....	134
6. Screening of polysaccharide coated microdevices for on-chip immunostaining	137
Abstract.....	138
6.1 Introduction.....	139
6.2 Experiments.....	141
6.2.1 Materials	141
6.2.2 Counting chamber fabrication and matrix preparation.....	141
6.2.3 Determination of surface roughness.....	142
6.2.4 Fluorescence imaging	143
6.2.5 Determination of matrix dissolution during sample inflow.....	143
6.2.6 Measurement of fluorescence profiles after sample inflow and on-chip immunostaining.....	144
6.2.7 Quantification of the fraction of released antibody during sample inflow and incubation	144
6.2.8 Determination of shelf life of matrix coated counting chambers.....	145
6.3 Results and discussion	145
6.3.1 Characterization of dry polysaccharide matrices with embedded antibody	146
6.3.2 Characterization of antibody distribution in the counting chambers after sample inflow	148
6.3.3 Evaluation of on-chip immunostaining.....	149

6.3.4 Thermal stability.....	150
6.4 Conclusion	152
References.....	153
7. Fully printed microfluidic devices enabling on-chip reagent storage and sample preparation for simple CD4 counting.....	155
Abstract.....	156
7.1 Introduction.....	157
7.2 Materials and methods	158
7.2.1 Inkjet printing of antibody-embedded composite layers.....	158
7.2.2 Characterization of printed layers	159
7.2.3 Frame printing and counting chamber assembly.....	160
7.2.4 Evaluation of on-chip immunostaining.....	161
7.2.5 Comparison of CD4 counts acquired from image cytometry tests with flow cytometry analysis	162
7.3 Results and discussion	162
7.3.1 Fluorescence recovery of fluorophore-labeled antibody in printed layers	162
7.3.2 Layer dissolution and antibody release during sample inflow.....	163
7.3.3 Optimization of on-chip immunostaining.....	164
7.3.4 Validation of CD4 counts obtained by image cytometry.....	166
7.4 Conclusion	167
References.....	169
Summary and Outlook	171
Samenvatting.....	177
Acknowledgement	183
List of publications	187
About the author	188

Introduction

Aim of the thesis

Point-of-care (POC) diagnostics are designed for use at or near the patient. Shifting complex diagnostics performed in centralized laboratories to robust and easy-to-use POC diagnostics can reduce cost and time needed for tests, allow early detection of diseases for prompt treatment and extend care to under-served populations. Due to these advantages, POC diagnostics has a high potential to address the challenges in patient-centered healthcare delivery including home testing (glucose monitoring), accident scene (blood gas/electrolyte detection), emergency (cardiac assessment) and field testing in low-resource settings (HIV, malaria and tuberculosis monitoring).

The success of POC diagnostics relies on the development of automated systems for rapid analysis of patient samples (e.g. blood). Rapid advancement in Lab-on-a-chip (LOC) technologies enables the integration of multiple analytical functions into self-contained microdevices for diverse biological assays (immunoassays, cellular analysis, nucleic acid analysis and clinical chemistry assays). In such devices, reshaping the conventional benchtop sample preparation into an on-chip format is essential. Over the last decade, various microfluidic concepts using sophisticated microstructure, employing regulated fluid and implementing external field have been developed to minimize the user's intervention in the sample preparation process. However, most development strategies still employ external equipment or partially requires manual operation for the sample preparation, very few concepts have met the requirements set by the Clinical Laboratory Improvement Amendments (CLIA), neither are they CLIA waived tests that can be applied into routine clinical practice.

In this thesis, the concepts in LOC technology and drug delivery are combined to develop hydrogel matrices integrated microfluidic chips, which enable controlled antibody release for on-chip immunostaining and cell counting ("InstantCount" concept). This robust on-chip sample preparation for cellular analysis in whole blood was achieved in a fully automated manner without any off-chip processing and accessory assistance, thus demonstrating its potential for POC applications.

Thesis outline

This thesis covers different LOC based sample preparation techniques in the point-of-care (POC) cellular analysis of whole blood, with an emphasis in development of immunostaining based cell identification and enumeration method to fulfill “InstantCount” concept.

Chapter 1 provides an overview of the latest on-chip cell separation and identification approaches, which are classified into two categories, based on physical and immune signatures, respectively. The advantages and limitations of each approach are discussed.

Out of those approaches, a new concept of a cell counting (CD4 counting: enumeration of CD4 positive (CD4⁺) T-lymphocytes per μL blood) assay by integrating hydrogel based controlled antibody release systems in microfluidic devices to realize on-chip staining is presented in the following chapters (Chapter 3-7). Various types of hydrogel were integrated in cell counting chambers and different techniques were developed to fabricate the chambers, which were tested for optimal immunostaining.

To demonstrate controlled antibody release in cell counting chambers, the release has to be quantitatively determined in time. In Chapter 2, the development of a real-time and *in situ* fluorescence imaging system to monitor the on-chip release of fluorophore-labeled antibody as a function of time in a flow chamber (which mimics the inflow of whole blood to the counting chamber for a CD4 counting assay) is discussed. This method offers the appropriate range of sensitivity and temporal resolution to track the on-chip release process. The detailed information of the method including sample characterization, imaging system setup, release platform establishment, signal validation and release quantification is described.

Utilizing this method, the on-chip release of fluorophore-labeled antibody from gelatin layers was studied in Chapter 3. Release kinetics determined in flow chambers

were shown to have a quadratic dependence on the layer thickness and can be described with a diffusion controlled release model. By influencing the preparation of gelatin layers, in terms of temperature, humidity and drying time, tailoring the release times of antibody from these layers was achieved. The in-depth understanding of the mechanisms governing antibody release in the flow chamber provides the necessary insights to successfully tune on-chip release for uniform antibody distribution and homogeneous cell staining in the counting chamber.

In Chapter 4, the development of an active on-chip antibody release process, which evolved from the above-mentioned passive release, is described. A temperature switched on-chip antibody release in the flow chamber was achieved. The release was initially prevented from matured inkjet printed gelatin layers due to extensive gelatin crosslinking and can be instantaneously triggered using a biocompatible temperature increase to dissolve the entire layer. This on-demand antibody release is beneficial to fulfill uniform and complete on-chip staining of CD4⁺ T-lymphocytes in the counting chamber, which is critical for immunostaining based image cytometry in our cell counting chambers with stopped-flow configuration. However, counting chambers containing printed gelatin layers suffer from short shelf life due to the ongoing conformational maturation of the gelatin matrix, which leads to a continuous change in release kinetics of the embedded antibody.

The success of a practical and affordable CD4 counting assay in low-resource settings not only requires external equipment/intervention-free on-chip sample preparation as discussed in the previous chapters, but also demands low-cost fabrication of these self-contained devices using simple technique. Chapter 5 reports the development of low-cost fabrication methods, with high-throughput capability, suitable for the production of POC CD4 counting chambers for use in resource-limited and low-income settings. Drop-on-demand printing methods were applied to fabricate the CD4 counting chambers in a fully automated manner. Mono-sized microbeads, suspended in UV-curable glue were printed on substrate slides using a piezo-actuated nanoliter dispenser. Subsequent addition of capping slides and UV curing of assembled

chambers using an automatic assembly setup constructed chamber compartments with defined area and controlled heights. Gelatin/antibody solutions were deposited by inkjet printing and resulted hydrogel matrix was used to enable on-chip sample preparation. The optimization of the key parameters of the printing methods for the development of counting chambers was performed. The performance of such fully printed CD4 counting chambers were tested in research and clinical settings.

To overcome the limitations of gelatin, due to its ongoing maturation, the feasibility of replacing gelatin by a polysaccharide as an alternative hydrogel matrix is explored in Chapter 6. Screening of eleven polysaccharides matrices including pure polysaccharides, polysaccharide blends and ionically crosslinked polysaccharide blends was based on the following aspects: topographical homogeneity (layer smoothness), antibody distribution, layer stability against sample wash-off and on-chip immunostaining. Out of these candidates, gellan was determined to be most promising. Counting chambers containing gellan layers show longer shelf lives, better thermal stability and more reproducible immunostaining than those containing comparable gelatin layers.

In Chapter 7, the alternative matrix material (gellan) was combined with the automated chamber fabrication methods, discussed in Chapter 5, to further explore POC CD4 counting assay. As described in Chapter 5, drop-on-demand printing methods were employed to fabricate both, chamber compartments and gellan/antibody layers for improved fabrication and testing reproducibility as well as reduced production cost. Simple but precise chamber design enabled hands-free on-chip sample preparation. The addition of trehalose to the gellan layer matrix allowed for improved antibody preservation before use. Desired antibody release was achieved for optimal on-chip staining. The obtained enumeration of CD4⁺ T-lymphocytes in whole blood shows a good agreement with the gold standard, flow cytometry.*

* Chapter 3-7 were written as independent research articles and there is overlap in introduction and method sections.

Chapter 1

On-chip sample preparation for point-of-care cellular analysis of blood

Xichen Zhang, Leon W. M. M. Terstappen, Markus Beck

This chapter is based on a book chapter:
Point-of-Care Diagnostics – New Progresses and Perspectives, IAPC-OBP (In Press)
ISBN: 978-953-56942-4-3

Abstract

Blood harbors information about the physiological and pathological conditions of the human body. The cellular analysis of blood samples plays a significant role in medical diagnostics. Conventional blood tests are performed in a centralized laboratory using calibrated equipment and operated by qualified personnel. Point-of-care (POC) diagnostic applications are generally expected to be compact and mobile, and at the same time to meet the performance of elaborate and often time-consuming protocols of established laboratory tests, which impedes the growth of POC applications. The emergence of Lab-on-a-chip (LOC) technology makes cellular analysis feasible at the POC. Here, we review the latest LOC based sample preparation techniques in the cellular analysis of blood samples for diagnostic purposes. We survey a variety of on-chip cell separation and identification approaches, which are classified into two categories, based on physical and immune signatures, respectively. Moreover, we discuss the advantages and limitations of each approach. Finally, we present an outlook of a fully integrated microdevice to achieve truly POC cellular analysis of blood samples.

1.1 Introduction

Blood flows throughout the whole body in a vigorous manner as the entire blood volume is circulated every minute. It is physiologically important as blood supports the homeostasis of the body by delivering oxygen and nutrients to every cell and transporting metabolic products between all types of tissues and organs. Additionally, the circulation allows cells of the immune system to travel to the desired site and perform immuno-surveillance in an efficient fashion. The clinical evaluation of the cellular composition of blood can provide important information on the body status and abnormal test results implicate the presence of disease or organ malfunctioning. Therefore, blood contains a wealth of information that greatly contributes to the diagnosis of our body.

Cellular analysis of blood is widely used for medical diagnostics. A complete blood count (CBC) surveys the cellular constituents of the whole blood with the quantification of each cell population (erythrocytes, leukocytes and platelets) and is routinely used to determine a person's health status. In a microliter of blood from a healthy donor, there are approximately 5 million erythrocytes (RBCs), 150,000-450,000 platelets and 4,000-11,000 leucocytes (WBCs) which can be further classified into 5 subpopulations: monocytes, lymphocytes, neutrophils, basophils and eosinophils.¹ An abnormal CBC often indicates the presence of a certain medical condition or disease, such as infection, inflammation, anemia and leukemia.² The CD4 count, measuring the number of CD4 positive (CD4⁺) T-lymphocytes per μL blood is widely used by medical professionals to monitor HIV progression.³ The identification of malaria parasites in RBCs and the quantification of parasitemia by examining blood smears is important to determine the patient prognosis.⁴ The presence of circulating tumor cells (CTCs) in the blood is associated with poor prognosis and the isolation and characterization of can be used to guide therapy.⁵

Sample preparation including separation, isolation and exposing cells to reagents in cellular analysis is crucial. Depending on the diagnostic needs, target cells should be either fully isolated or exclusively distinguished prior to further characterization for improved specificity and sensitivity, since irrelevant cells in the blood might alter the condition of target cells or interfere with the analysis. The extreme heterogeneity in

blood cell populations creates challenges in the sample preparation for cellular analysis in blood.

Most cellular analysis is performed in centralized laboratories, where sample preparation has to follow strict protocols with the aid of high-end external equipment. Accessibility of these tests at bedsides, disaster areas and low resource settings are challenging.

To address these challenges, microfluidic based Lab-on-a-chip (LOC) technology proposes practical solutions. The innovative concept of miniaturizing conventional sample preparation into a microdevice is very attractive to perform cellular analysis of blood samples at the POC. Compared with conventional off-chip sample preparation, the downscaled dimensions of microdevices allow for reduced blood volume, thus minimizing the discomfort and adverse effect of blood drawing, as well as reducing consumption of reagent, energy and time. In addition, the miniaturized size renders increased portability to enable sample preparation performed on site where it is needed. The integration of complex bench-top operations into a self-contained microdevice realizes a (semi-)automated processing step, greatly simplifying the sample preparation and reducing manual errors for enhanced reproducibility.

This chapter summarizes the latest progress in LOC technology, with a focus in the development of sample preparation for the cellular analysis of blood samples. An overview of different approaches which enable either partial or complete on-chip sample preparation is presented. The approaches of on-chip cell separation and identification are grouped into two categories: a reagent-free strategy based on the diversity in physical signatures and a reagent-biomarker interaction strategy based on the difference of immunoaffinity. The principle of each approach and the details of specific microfluidic design are elaborated, followed by the demonstration of diagnostic applications for relevant medical conditions. The evaluation (simplicity, specificity and sensitivity) of each approach is also reviewed. Finally, some recommendations on improving the current cell sampling techniques to achieve truly POC cellular analysis are given.

1.2 Reagent-free cell separation based on physical characteristics

The heterogeneity of cells in blood is reflected in their diverse physical properties. In the light of physical variation among different cell populations, numerous separation principles have been developed and implemented in LOC systems. Cell size, shape, deformability, density and dielectric property are commonly used parameters to distinguish target cell populations. Physical separation based on these parameters does neither consume biochemical reagents for cell labeling nor for cell capture, as are so-called label-free approaches, thus achieving simple and reproducible on-chip sample preparation. In this section, recent progress in physical separation approaches is summarized.

1.2.1 Filtration

Cell size is the one of most noticing properties when examining a blood smear with a microscope. Mechanical filtration is an effective approach to separate cells based on distinctive size. A well-established application is the separation of WBCs and RBCs in whole blood.⁶⁻⁹ Bench-top filtration concepts using fibrous membranes (nitrocellulose⁷, polycarbonate^{8,10}, polyacrylamide¹¹) can be simply down-scaled and implemented into microdevices. In such devices, whole blood flows perpendicular to the membrane comprising a magnitude of pores with precisely controlled size, which allow the passage of cells smaller than a critical size and retain larger counterparts. In addition to miniaturized membranes, obstacle microarrays can also enable an excellent sieving effect. A variety of array structures in terms of micropillars/microposts¹²⁻¹⁴, microweirs¹⁴, microtraps¹⁵ have been designed and fabricated. By tailoring the dimension, geometry as well as the arrangement of obstacles, the desired size cutoff can be achieved. Sample throughput can be enhanced by increasing the number of filtration units.

Although mechanical filtration is a very simple, versatile method that allows for high-throughput sieving, there are still two major challenges that have to be addressed. The first challenge is clogging, which is due to the dead-end filtration scheme (perpendicular sample flows to membranes or obstacle microarrays in a single

channel, Fig. 1.1ia). Saturation of filtration units leads to decreased filtration efficiency and impractical regeneration of these units results in problems for continuous sample processing. The second challenge is regarding cell viability. Cells suffer from a risk of damage when moving under high flow rate or squeezing through a filtration unit. Elevated shear stress and concentrated tensile stress compromise the viability of cells, reducing the potential for subsequent cell culture or molecular analysis.

Cross-flow filtration has been developed to overcome the clogging issue. (Fig. 1.1ib) It consists of a main channel and one or multiple side channels, creating more exits for sample fluid. After being introduced, sample fluid can be directed either into side channels through microfilters or retained in the main channel. Cells suspended in blood flow at a size-dependent tangential rate in the stream. Smaller cells are extracted into side channels through microfilters and travel to the corresponding outlets, while cells that are too large to pass through the microfilters are swept to the outlet of the main channel, without clogging the flow path.⁹ Lee and coworkers developed a simple cross-flow filtration system with two inlet ports and two outlet ports. Buffer solution and blood sample are introduced into a channel and encounter an inclined silicon microfilter array where larger cells are diverted into a secondary channel and smaller cells travel through the filters. With appropriate hydrodynamic pressure for both streams, the device was able to realize a 74% recovery of fetal nucleated RBCs and a 46% depletion of adult RBCs from cell model mixtures.¹⁶ (Fig. 1.1ii) In addition to the inclined orientation in the flow path, microfilter array can also be arranged parallel to the flow path in the main channel. Chen et al. and Sethu et al. developed cross-flow filtration systems with one main channel in the center and two side channels in a parallel arrangement. Micropillar¹⁷ and microweir¹⁸ arrays are located at the interfaces between the main channel and lateral channels, enabling cross flows. With the optimized flow dynamics, WBC enriched and depleted blood fractions can be harvested from the outlets of the main channel and lateral channels, respectively. (Fig. 1.1iii) In order to improve efficiency and purity in WBC enrichment, VanDelinder and coworkers developed a microdevice with a deep main channel for the passage of WBCs and an array of orthogonal, shallow side channels for the entrapment of RBCs.¹⁹ (Fig. 1.1iv) As whole blood advances in the main channel, a perfusion flow

through the side channels gradually washes away RBCs. This method achieved 4000-fold enrichment and 98% retention of WBCs from whole blood. Reverse-flow filtration provides another concept to prevent clogging and increase the life time of filtration units. McFaul and coworkers fabricated a microratchet array and employed an oscillatory flow to selectively regenerate the pores blocked by larger and less deformable cells.²⁰ (Fig. 1.1v)

To preserve cell viability, a 3D filtration system with two porous PDMS layers has been developed to provide a gentle capture to preserve cell integrity.²¹ (Fig. 1.1vi) When cancer cells are trapped in the filtration units from the top layer, the bottom layer provides support in the opposite direction to compensate tensile stress on the cells. More than 85% of the captured cancer cells were reported to remain intact.

Fig. 1.1: Schematic presentation of different filtration schemes and examples. i) Dead-end and cross-flow filtration. ii) Separation of fetal nucleated RBCs (FNRBCs) and adult RBCs by a micropillar array inclined to flow direction. When the parallel flows of cell suspension (blue dash lines) and buffer (solid black lines) encounter the filter array, the smaller adult RBCs penetrate through the filters; whereas larger FNRBCs are diverted. iii) Separation of RBCs from whole blood by a microweir array parallel to flow direction. Insert shows the 40 mm × 2.5 mm sieve structure and the arrangement connecting the main channel to lateral channels. iv) Depletion of RBCs from whole blood by a microchannel array parallel to flow direction. The horizontal main channel is 25 μm deep for WBC enrichment, and the vertical channels are 3 μm deep allowing the diversion of RBCs. Image a) to h) are the partial regions of the channel array at distances from 0 to 16.8 mm away from the beginning of the separation network with increments of 2.4 mm. v) Ratchet cell sorting. Smaller and more deformable cells can squeeze through the funnel constrictions during forward flow. However, they are unable to pass back through the funnels when the flow direction is reversed periodically to unclog the filter. vi) Filtration using a 3D microfilter device. Inset shows the applied forces on a trapped cell. F_L : force caused by the fluidic flow pressure. F_S : supporting force from the bottom. F_T : tensile stress force on the plasma membrane. i) ref. 9, copyright 2007 Springer; ii) ref. 16, copyright 2010 Elsevier; iii) ref. 18, copyright 2006 Royal Society of Chemistry; iv) ref. 19, copyright 2007 American Chemistry Society; v) ref. 20 copyright 2012 Royal Society of Chemistry; vi) ref. 21 copyright Springer.

1.2.2 Deterministic lateral displacement

Deterministic lateral displacement (DLD) is another well-established cell separation approach on the basis of size in continuous flow conditions. This approach was first developed by Huang and coworkers who applied this technology to sort particles with different sizes.²² A recent review provides in-depth understanding of the mechanism underlying this size-based particle separation.²³ As illustrated in Fig. 1.2i, a microfluidic channel hosts a specific arrangement of microposts, which splits the sample flow into different streamlines and determines the trajectory of accommodating cells. Cells suspended in the fluid are distributed in different streamlines.²⁴ When a cell is smaller than a critical diameter and its center falls within the width of the first streamline, a drag force will be dominant to keep the cell unperturbed to follow a zigzag route in the first streamline. Meanwhile, a larger cell will fall into an outward streamline, which deflects from the first one, thus becoming displaced from the smaller counterpart. By shifting each succeeding post row laterally at a predefined distance from its predecessor, periodical deflection in cell path will be created, leading to an amplified displacement in cell trajectory and achieving cell separation. In the review, McGrath and coworkers have proposed a toolbox, in which the influence of micropost array design (size, shape, orientation and spacing) on the critical size for cell displacement and the consideration of microfluidic phenomena (profile of laminar flow, diffusion and fluidic resistance) on the performance of DLD are summarized in detail. This toolbox is very beneficial as a design aid to develop tailored microdevice to separate cells of specific sizes. Cancer cells spiked into whole blood have been recovered from the mixture with more than 85% efficiency using DLD.²⁵ Advanced DLD designs are capable to separate whole blood into multiple fractions²⁴, for instance the fractionation of platelets with different sizes by employing a series of different micropost arrays.²⁶ (Fig. 1.2ii and 1.2iii) Each array has a slightly different critical diameter. Using this method, size discriminating resolution was enhanced step by step and a successful separation between WBCs, RBCs and platelets was achieved.

The concentration of the cells in the sample fluid determines the efficiency of cell separation. In whole blood samples, the dense concentration of RBCs will increase the

likelihood of RBC contamination in outward streamlines due to the overcrowded RBC population in the first streamline. Moreover, non-specific interactions between microposts and suspended cells will increase flow resistance and finally lead to clogging, especially for blood samples with dense cell concentration. Therefore, blood samples with high cell concentration need to be diluted prior to processing. Since DLD is designed for separating rigid and spherical particles, another challenge of efficient blood cell separation lies in the non-spherical shape and deformability of RBCs.²⁷ Although improved efficiency has been demonstrated, the greatly increased complexity in microarray fabrication and largely reduced throughput outweigh the benefit.^{28, 29}

Fig. 1.2: i) Schematic presentation of the DLD principle. The orientation of streamlines are induced as a consequence of lateral micropost shifting and the distinctive trajectory of particles falling into different streamlines. ii) Left, A DLD device designed to separate WBCs from RBCs and platelets. A cascade of 13-section post arrays with different critical diameters and spacings. Right, The predicted size dependent displacement of cells, which travel through the post arrays. The lateral position of the blood injection point at the top of the device is shown. Three exit channels at the bottom of the device are used to collect the fractionated blood. iii) Left, Diagram of ideal particle paths in the platelet fractionation device. WBCs are moved to the left by Array 1 into a channel that runs parallel to but is not in connection with the platelet fractionation array, Array 2. It is composed of a series of 11-section post arrays, each with increasing critical particle size for increasing lateral displacement. Right, Micrographs of various cells in the device at three locations: whole blood injection at inlet (upper), WBCs separation at Array 1 (left) and platelet fractionation at Array 2 (right). i) and ii) ref. 24, copyright 2006 National Academy of Sciences, U.S.A; iii) ref. 26, copyright 2008 Royal Society of Chemistry.

1.2.3 Pinched flow fractionation/Hydrodynamic filtration

Pinched flow fractionation and hydrodynamic filtration have shown potential in size-dependent cell separation. Their operation principles are similar. When a sample fluid with low Reynolds number is traveling in a microchannel, the trajectories of suspended cells are determined by the paths of streamlines, which they are falling into.³⁰ The distribution of cells in streamlines is closely related to cell size and flow profile. Therefore, tuning the flow profile via the optimization of flow rate, different design in the geometry of microchannels and the arrangement of inlet and outlet can achieve size-dependent cell sorting.

Pinched flow fractionation was first proposed by Yamada and coworkers for precise particle separation.³¹ During the operation, two laminar streams were pumped through a pinched segment before entering a broadened channel. One stream was diluted blood and the other was buffer solution, which flowed at a higher rate to push blood cells against the sidewall in the pinched segment, resulting in an alignment of the cell mixture. Smaller cells were distributed in the streamline closer to the sidewall than the larger counterparts. At the boundary between the pinched segment and the broadened channel, a transition in flow profile occurred. Dramatically spreaded streamlines amplified the position difference between smaller and larger cells in the pinched segment and led to a cell dispersion perpendicular to the flow direction according to their sizes in the broadened channel. The broadened channel was then branched into multiple outlets with optimized arrangement. Enriched RBCs were harvested from a predetermined outlet of a pinched microchannel.³² (Fig. 1.3i)

Hydrodynamic filtration has been demonstrated by Yamada and Seki using devices that consist of a straight main channel with a single inlet and a number of perpendicular branched channels.³³ By pumping the sample fluid along the main channel and withdrawing it from branched channels, cells are aligned along the sidewall of the main channel. Smaller cells distributed adjacent to the sidewall, suffer less flow resistance and enter branched channels upstream. Larger cells are still far away from the sidewall, thus being forced to flow straight. Every time the fluid is drawn to the branched channel, larger cells are brought closer to the sidewall and eventually flow into the branch channels. This channel network enables displaced cell

alignment against the sidewall of the main channel based on the size, therefore offering a promising solution of WBC enrichment from whole blood. (Fig. 1.3ii)

Desired flow rate is crucial to manipulate the trajectory of cells in the channel. This demand of external pumping system increases the complexity for the applications. High cell concentrations result in reduced sorting efficiency and therefore necessitate a dilution step prior to sorting.

Fig. 1.3: i) Schematic presentation of particle concentration and classification in a microchannel having multiple branch points and side channels. ii) Schematic presentation of pinched flow fractionation. i) ref. 32, copyright 2005 Royal Society of Chemistry; ii) ref. 33, copyright 2005 Royal Society of Chemistry.

1.2.4 Inertial microfluidics

After being neglected for many years, the contribution of inertia to microfluidic phenomena received a lot of attention in the last decade, as experimental discoveries together with theories explaining the observed phenomena overturned the traditional understanding that microfluidics is associated with negligible inertia. Instead, inertia becomes apparent in a fluid with high Reynold number and significantly contributes to microfluidic phenomena.^{34, 35} Di Carlo explicitly elaborated a dominant effect, inertial migration of particles, which is widely exploited for cell separation.³⁶ Such migration is induced by inertial lift forces intrinsic to cell motion in confined channel flows. In Poiseuille flow, two opposing lift forces act perpendicular to the flow direction on a moving cell. The wall induced lift drives the cell away from the channel wall with a force decreasing with the distance from the channel wall. In contrast, the shear gradient induced lift pushes the cell towards regions of higher shear gradient, i.e. towards the channel wall in a parabolic velocity profile of Poiseuille flow. Interaction of the two forces on a given type of cells results in an equilibrium at a focused streamline, which defines a characteristic trajectory for effective cell separation.³⁶ (Fig.

1.4i) Key parameters to control inertial lift equilibrium and cell focusing are channel geometry, flow rate, cell size and deformability. After reviewing these parameters for tunable cell separation, Amini and coworkers proposed tips to design appropriate microfluidics to sort cells with different properties (shape, size and deformability) based on the latest development of inertial microfluidics.³⁷ With respect to microfluidic design, a multitude of geometries, in term of straight^{35, 38, 39}, curved⁴⁰, spiral⁴¹, pinched³⁴ and alternative expansion and contraction edges⁴² have been developed for cell sorting. (Fig. 1.4ii-vi) Several cell sorting applications based on these principles have been demonstrated, for example the isolation of CTCs from the other cell populations in whole blood.⁴² The deformation of cells, in particular CTCs under high shear stress, was reported to have an impact on cell focusing in the channel, while the influence in separation efficiency was still inconclusive.^{34, 41} To pursue efficient separation of cells that are prone to deform, the difference in deformability was also exploited in inertial microfluidics to enrich CTCs from diluted blood samples.³⁹ With the aid of size and deformability as dual distinctive features, the fractionation of diluted blood into RBCs, platelets, peripheral blood mononucleated cells and granulocytes was achieved by a real-time deformability cytometry.³⁸ For further increased efficiency and purity, the size and deformability based separation was cascaded in series to achieve a 100-fold enrichment of platelets from diluted blood.⁴⁰ In high flow rate conditions, sensitive cells subject to strong inertial interaction are seriously stressed. The risk of cell damage questions the validity of subsequent cellular analysis after separation. To address this issue, a soft inertial microfluidic system developed by Wu and coworkers employed a sheath flow to reduce shear stress and protect fragile cells during separation.³⁵ *Escherichia coli* (*E. coli*) was successfully isolated from diluted blood and the viability of *E. coli* was verified by comparing the protein content from cells before and after separation.

Similar to hydrodynamic filtration, the performance of inertial microfluidics is highly dependent on the precise flow control. This strict requirement of external pumping system may narrow down the scope of applications, in particular for adverse operation condition and low-resource settings. Moreover, the sample concentration is another limitation. Whole blood with dense cell concentration has to be diluted in a

pretreatment step to maintain the separation efficiency, thus reducing the value in the simplicity of this approach.

Fig. 1.4: Principle of particle separation by inertial microfluidics (i) and layout of inertial microfluidics with straight (ii), curved (iii), spiral (iv), pinched channels (v) and the channel with alternate side edge (vi). i) ref. 36, copyright 2009 Royal Society of Chemistry; ii) ref. 35, copyright 2009 Royal Society of Chemistry; iii) ref. 40, copyright 2008 American Society of Chemistry; iv) ref. 41, copyright 2012 Royal Society of Chemistry; v) ref. 34, copyright 2011 Royal Society of Chemistry; vi) ref. 42, copyright 2013 American Chemistry Society.

1.2.5 Dielectrophoresis

Cells in the blood stream are a heterogeneous population of biological particles. Each subpopulation possesses intrinsic dielectric properties, which is closely related to membrane capacitance and cytoplasm resistance.^{43, 44} Dielectrophoresis (DEP) will occur, when exposing blood into a non-uniform electric field. According to the frequency of the field, polarized cells can either exhibit positive (attraction to high electric field) in low conductive fluid or negative DEP^{45, 46} (repulsion from high electric field) in physiological media with high conductivity. This selective cell response makes dielectrophoresis a powerful tool to manipulate the dynamic behavior of cells in blood for the separation and sorting purpose.

Incorporating DEP into microdevices can be easily achieved. Well-developed Micro Electro Mechanical Systems (MEMS) technology enables a straightforward implementation of electrode and exquisite control of electric field in microdevices. DEP can be implemented in a very efficient way in microfluidics, as the small dimension in microfluidic devices allows for the generation of intense electric field gradients using rather low voltages.

Microfluidic DEP for cell sorting can be grouped into field-flow fractionation, i.e. the deflection of cells from their streamlines and the capture of cells by the dielectrophoretic force. DEP field-flow fractionation featured with continuous flow has a striking advantage for high-throughput sample preparation. The working principle is well elaborated by Lee et al.⁴⁷ and Yang et al.⁴⁸ When cells flow through a straight microchannel subject to an electric field, they experience three acting forces: DEP force, hydrodynamic force and gravity. By tuning the electric field (voltage and frequency) in a custom manner, the desired equilibrium of DEP force and gravity levitates cells with varying dielectric properties and densities to distinct streamlines. The parabolic profile induced velocity variance will allow cells to be eluted from the microchannel at different times. In addition, split downstream microchannels will harvest cells spatially separated in the main stream. One of the pioneering works achieved an elegant WBC 3-part differentiation by this technique.⁴⁸ Recently, the separation of CTCs⁴⁹⁻⁵¹ (Fig. 1.5i), platelets^{52, 53} and pathogens (bacterial cells^{54, 55}, yeast cells⁵⁶) from whole blood has been demonstrated with over 90% recovery rate.

In addition to the cell fractionation in a flow, DEP assisted cell trapping is to realize cell separation in a still fluid. In this concept, electrode microarrays function as steric traps to attract cells with positive DEP response. Different patterns of microarrays, in terms of linear post⁵⁷, interdigitated^{58, 59} and spiral were developed. (Fig. 1.5ii-iv) This approach is suitable for submicron organism separation in blood samples. *P. falciparum* infected RBCs⁴⁴ and *E. coli*⁵⁸ were demonstrated to be captured from blood and released upon electric field turn-off.

With regarding to the limitation, both approaches require exquisite electric control and flow control, thus they appear to be impractical for low-resource settings.

Fig. 1.5: Microfluidics DEP devices. (i) DEP field-flow fractionation cell sorter for CTC isolation. (ii) Top, Linear electrode array illustration; bottom, image of single cell capture. (iii) Interdigitated electrode array with cancer cells trapped at tips after blood removal. (iv) Distinct distribution of normal erythrocytes (edge of electrode) and *P. falciparum* infected erythrocytes (center of the spiral). i) ref. 49, copyright 2012 American Institute of Physics; ii) ref. 57, copyright 2002 American Society of Chemistry; iii) ref. 59, Copyright 1995 National Academy of Sciences, U.S.A; iv) ref. 44, copyright 2002 Royal Society of Chemistry.

1.2.6 Lab-on-a-disk

Cell density is another common physical property to separate different cell populations in blood. Centrifugal approaches are particularly applicable in the extraction of blood constituents (erythrocytes and buffy coat) from different sediment layers. The lab-on-a-disk approach successfully miniaturizes the bench-top centrifugation protocol into an on-chip sampling step. Compared with the aforementioned Lab-on-a-chip systems, the centrifugal lab-on-a-disk platform offers striking advantages in flow control. Instead of pressure-driven flow, sample handling in the lab-on-a-disk platform is simply driven by a conventional spindle motor, thus eliminating the need of elaborate pumping system. Centrifugal actuation independent of fluid properties such as pH or conductivity renders robustness in handling heterogeneous blood samples. The recent advances in the lab-on-a-disk platform for cell analysis were well summarized by Burger et al.⁶⁰ Separation of the entire cellular constituent in whole blood can be readily achieved by plasma sedimentation in the lab-on-a-disk platform employing straight⁶¹, curved⁶², azimuthally inclined⁶³ and spiral channels⁶⁴. Recently, more and more attention has been drawn to selective separation of target cell populations from whole blood. Density gradient configuration has been employed to separate WBCs from RBCs⁶⁵ or to enrich basophils⁶⁶ from peripheral human blood.

Although the lab-on-a-disk platform exhibits broader compatibility with different sample fluids and less constraints on external equipment, it still has its own limitations, in terms of unidirectional flow and difficulty in extracting a very small cell population.

1.3 Biomarker mediated cell identification and sorting based on immune characteristics

Compared with physical properties, cellular immunoaffinity is more potent to improve specificity in cell separation and achieve explicit cell identification. Biomarkers at cell membranes, e.g. surface antigen, are the immunological fingerprint of a cell population. The exclusive key-lock interaction between biomarkers and relevant biomolecules can be employed to identify and sort different cell populations in blood.

1.3.1 On-chip immunostaining and cell identification

The current gold standard for cell identification based on immunoaffinity is flow cytometry. In this technology, cells pre-stained with one or several types of fluorophore-labeled antibody are identified by passing through excitation laser beams in a serial manner and detecting scattered and fluorescent light with various optical detectors. Traditional flow cytometers are bulky instruments offering a multitude of options, and therefore often require highly skilled operators as well as bench-top sample preparation.

Recent developments in microfluidics make promising progress in enabling cytometry technology for point-of-care blood diagnostics.⁶⁷ Several of these devices use counting chambers with integrated reagents for on-chip immunostaining and a portable fluorescence imager for the detection of stained cells.^{68, 69} In our approach (described in details in the following chapters of this thesis)⁷⁰, unlysed whole blood flows into the chambers driven by capillary forces, and antibodies are released into the sample after the inflow has stopped. Fluorescence imaging after 30 min incubation yields sufficient contrast between cells and background without the need for a washing step. The prerequisite of on-chip staining in such conditions is homogenous mixing of blood and integrated reagents. To achieve even distribution of reagent in blood, hydrogel matrix is incorporated in the chamber to release embedded reagents in a controlled manner upon the contact with blood. Based on this principle, we have developed a self-contained chip with on-chip sample preparation for CD4 counting.⁷⁰ One side of the interior surface in the chamber is covered with a manually deposited dry gelatin layer, which contains fluorophore-labeled antiCD3 antibody (α CD3) and

antiCD4 antibody (α CD4). The chamber is filled with whole blood through capillary action. The well-defined chamber height and image area of the fluorescence imager determine the blood volume. The principle of on-chip sample preparation lies in a passive release scheme. Diffusion controlled antibody release only starts when a certain degree of gelatin swelling is reached after blood inflow. This release delay is beneficial to prevent antibody wash-off in the chamber. (Fig. 1.6i) By tuning the preparation of gelatin layers (thickness and degree of physical crosslinking), optimal release delay was achieved to maintain homogeneous antibody distribution in the chip, meanwhile ensuring more than 80% antibody fractional release within 10 min for rapid and sufficient immunostaining of target cells.⁷¹ (Fig. 1.6ii) The stained cells are imaged and the analysis of the acquired image yields the cell count. Thermally activated release in the same chip format was employed to demonstrate temperature switched immunostaining.⁷² In this work, gelatin/antibody layers were produced by inkjet printing, which turns the labor-intensive preparation to a semi-automatic process and improves layer reproducibility. The layers are subject to an appropriate maturation process to increase the degree of physical crosslinking in gelatin, resulting in a dense matrix network to trap antibody during blood inflow. On-demand antibody release after inflow can be triggered by heating the layer over the sol-gel transition temperature. Elevated temperature leads to gelatin dissolution for complete and rapid antibody release. (Fig. 1.6iii) Such concept of initially preventing release (during inflow), followed by an instantaneous release on demand successfully achieved uniform and complete on-chip immunostaining. Compared with the passive release scheme, this active release scheme offers better temporal control for the on-chip sample preparation and can be beneficial for other biological assays, which requires a series of mixing steps. A similar concept was reported by Jin and coworkers⁷³, who achieved on-chip antibody release via the dissolution of polyvinylpyrrolidone (PVP) nanofibers for *E. coli* identification. Another approach to trigger the release of reagents for on-chip sample preparation is pH responsive release. Mortato and coworkers immobilized poly(methacrylic acid) microgels with embedded fluorophore-labeled antibody in a microfluidic channel.⁷⁴ The filling of whole blood with physiological pH induces maximum swelling of the microgel, thus enabling the

release of reagent to stain T-lymphocytes and B-lymphocytes on chip, however without the possibility to influence the timing of the release externally.

On-chip sample preparation enabled by controlled reagent release greatly simplifies the conventional staining protocol including metering, pipetting, vortexing and rinsing. It does however require reagent release kinetics that is largely independent of environmental conditions such as temperature and humidity, especially for applications in low-resource settings.

Fig. 1.6: i) Schematic presentation of on-chip sample preparation for CD4 count. ii) Tuning antibody release kinetics by different layer preparation. iii) Influence of delayed release on the homogeneity of antibody distribution. (upper) Layer maturation induced delayed release and temperature triggered release. (lower) i) ref. 70, copyright 2012 Royal Society of Chemistry; ii) ref. 71, copyright 2016 Royal Society of Chemistry; iii) ref. 72, copyright 2016 American Chemistry Society.

1.3.2 On-chip immunomagnetic cell separation

In addition to fluorophores, magnetic particles are also widely used to label antibodies that can recognize specific surface antigens. The coupling of antibody coated magnetic particles against target cells has been reported to achieve highly selective

immunomagnetic separation from bulky cell mixtures.⁷⁵ Implementing this separation scheme into microdevices can further enhance the separation purity and efficiency due to increased magnetic field gradients.

A one-step immunomagnetic separation of T-lymphocytes from whole blood was realized on a simple microchannel.⁷⁶ Anti-CD3 coated magnetic beads are first introduced into the channel and accumulated at a precise location where permanent magnets are placed. These formed bead beds serve as a regional binding site to capture T-lymphocytes in the upcoming blood flow. (Fig. 1.7i) In a similar concept, CTCs spiked whole blood is pre-stained with anti-EpCAM functionalized magnetic particles and flows through a microchannel, which is closely above a magnetic array. By tuning the flow rate, the CTCs were separated from blood and deposited at the bottom of the channel with 85 to 90% capture rate.⁷⁷ In a more complex format, a planar array of magnetic stripes in a microchannel was designed.⁷⁸ (Fig. 1.7ii) Whole blood is injected into the microchannel and flows over the magnetic array. WBCs coupled with anti-CD45 functionalized magnetic particles are clearly shown to deviate from the flow direction and proceed along the orientation of magnetic strip. However, the track of RBCs is not deflected. This distinct flow pattern allows for excellent WBC separation. Saliba and coworkers developed a hexagonal array of magnetic ink patterned at the bottom of a microchannel. After the filling of antibody coated magnetic beads into the channel, external vertical magnetic field is applied to align free-moving beads into columns, which are anchored on top of the ink dots.⁷⁹ (Fig. 1.7iii) This self-assembled 3D magnetic trap array was successfully demonstrated to capture leukemia cells⁷⁹ and CTCs⁸⁰ from whole blood.

A minor concern of this approach lies in the interference from RBCs. In deoxygenated blood, the reduced form of iron-bearing hemoglobin may render RBCs paramagnetic.⁸¹ Although the intrinsic magnetic susceptibility of RBCs is much smaller than that of target cells coupled by antibody mediated magnetic particles, the purity of target cells isolated from whole blood has to be further verified.

Fig. 1.7: Magnetic activated cell capture. (i) Immunomagnetic separation of T-lymphocytes using Y-intersection device. (ii) A single tagged fluorescing leukocyte at different times tracking a magnetic stripe (right) and unlabeled erythrocytes (left) following fluid direction (white arrow). (iii) Alignment and anchorage of antibody coated magnetic beads for leukemia cell capture. i) ref. 76, copyright 2004 Royal Society of Chemistry; ii) ref. 78, copyright 2004 American Institute of Physics; iii) ref. 79.

1.3.3 On-chip immunocapture

Specific adhesion of cells on a surface functionalized with a given ligand provides another strategy to address poor selectivity and low efficiency in physical-based cell separation. Adhesion based microdevices for cell separation have gained increasing attention due to their specificity and reliability as well as their label free operation. The outcome of cell capture in microdevices is influenced by the employment of ligand molecule, the device design and the applied flow rate. The affinity between ligand molecule and cell determines the strength that cell can adhere to the surface. The shear stress induced by the geometry of microchannel and flow rate diminishes cell adhesion.⁸² Therefore, a successful application demands a thorough optimization for

all three factors.

Antibody is the most common ligand molecule due to the well-exploited antigen-antibody interaction for cell capture. Epithelial cell adhesion molecule (EpCAM) is a diagnostic marker for a variety of cancers. Negrath and coworkers constructed a microchannel hosting an anti-EpCAM immobilized micropost array to capture CTCs from whole blood.⁸³ (Fig. 1.8i) A 99% detection rate and more than 50% capture purity were reported. In addition to relying on a single type of antibody for cell capture, a panel of antibody was immobilized on the bottom surface of a microchannel to maximally match the phenotype of circulating endothelial progenitor cells for higher capture efficiency in blood.⁸⁴ Diverse concepts were developed for CD4 counting based on the capture of CD4⁺ T-lymphocytes.⁸⁵⁻⁸⁸ Wang and coworkers captured all CD4⁺ cells in a microtrap array and labeled captured cells with α CD3-enzyme conjugates in a sandwich manner for chemiluminescence detection.⁸⁵ (Fig. 1.8ii) Cheng and coworkers excluded the influence of monocytes in CD4 count by a serial capture of monocytes and CD4⁺ T-lymphocytes on a planar surface.⁸⁷ By using the same α CD4 for immobilization, micropost arrays were found to be more efficient than a planar surface in the same microchannel in capturing CD4⁺ T-lymphocytes due to increased surface areas.⁸⁸ Apart from microarray and channel surface, peptide-functionalized alginate hydrogel was also employed to capture cells in a microdevice.⁸⁹ A striking advantage is that the reversibly crosslinked alginate can be dissolved to release captured cells for further analysis. Microbeads are another carrier for antibody immobilization, a group of densely packed microbeads in a microchannel was able to capture *E. coli* with excellent efficiency from 91% to 95%.⁹⁰ (Fig. 1.8iii)

Fig. 1.8: Adhesion based cell capture. (i) Overall and detailed picture of CTC isolation system. a, A workstation; b, A CTC chip with microposts etched in silicon; c, Whole blood flowing through the device; d, Lung cancer cell captured by antibody coated micropost array. (ii) Schematic diagrams and pictures of a microdevice hosting antibody coated microtrap array for capturing CD4⁺ T-lymphocytes using chemiluminescence based detection. (iii) Schematic of the microchannel packed with antibody coated glass beads for *E. coli* capture. The thickness step of the channel ensures the monolayer formation and prevents beads being washed away. i) ref. 83, copyright 2007 Nature publishing; ii) ref. 85, copyright 2010 American Chemistry Society; iii) ref. 90, copyright 2010 Springer.

Aptamers, short chain oligonucleotides, recently receive growing attention as they shows great potential to selectively target desired cell types.⁹¹ The immobilization of aptamer onto the surface of a microchannel is realized via biotin-avidin interaction. An interesting microdevice with aptamer functionalized microwell array was tested to capture cancer cells from whole blood.⁹² By optimizing the size of the microwells, single-cell occupancy was obtained as high as 88%. Three different aptamers: sgc8, TD05 and Sgd5 immobilized onto the planar surface of microchannels were employed to target CCL-119 T-cells, Ramos cells and Toledo cells, respectively in whole blood. A 135-fold enrichment was obtained with over 96%

purity, suggesting profound specificity and selectivity in cell separation.^{93,94} Thanks to this high yield in cell enrichment, aptamer based microdevices were capable to detect the chemiluminescence produced by leukemia cells down to 10 cells/ μ l in the blood sample.⁹⁵

External pumping enabled precise flow for desired shear stress is of great importance for efficient capture. Therefore, the availability of immuno-capture based cellular analysis is limited only to well-equipped research environments.

1.4 Outlook

Over the last decade, fast-paced advancement in LOC technology has driven the development of a diversity of on-chip sample preparation concepts for cellular analysis of blood samples. Few successful concepts are applied into practice for routine POC blood tests. The underlying reason is that the potential of POC testing is not fully reached by the current concepts. An ideal LOC device for POC blood tests should be able to take whole blood in and provide the result out. In addition, the entire process is completely automated and accessory-free. There are two issues holding backing the application of current concepts. First of all, the required accessories for fluid actuation and (electric and magnetic) field implementation prevent the use of on-chip cell separation and sorting at the POC. Second, current LOC devices for cellular analysis do not meet the need for comprehensive blood tests, in particular the genetic analysis of small subpopulations of cells, such as for the analysis of CTCs and foreign pathogens in blood samples. To overcome the limitation, the following issues need to be addressed. From the operational point of view, a microdevice should enable self-regulated fluid delivery (e.g. capillary driven) and on-chip sample-reagent mixing (e.g. controlled reagent release) to secure (semi)-automatic sample preparation. From the functional point of view, successive preparation and analysis steps may require segmented compartments (e.g. for cell identification and capture, cell lysis, biomolecular sensing), which should be implemented into microdevices to fulfill the requirement of multi-step sample preparation for blood tests. In other word, the simplicity of a lateral flow assay, one of the most successful POC solutions, has to be combined with the performance of advanced LOC technology.

References

1. K. E. Barrett, S. Boitano, S. M. Barman and H. L. Brooks, *Ganong's Review of Medical Physiology, McGraw-Hill Medical*, New York, 2010
2. B. J. Bain, *N. Engl. J. Med.*, 2005, 353, 498-507.
3. D. S. Stein, J. A. Korvick and S. H. Vermund, *J. Infect. Dis.*, 1992, 165, 352-363.
4. N. J. White, *N. Engl. J. Med.*, 1996, 335, 800-806.
5. W. J. Allard and L. W. M. M. Terstappen, *Clin. Cancer Res.*, 2015, 21, 2883-2885.
6. A. Bruil, W. G. van Aken, T. Beugeling, J. Feijen, I. Steneker, J. G. Huisman and H. K. Prins, *J. Biomed. Mater. Res.*, 1991, 25, 1459-1480.
7. X. Yang, O. Forouzan, T. P. Brown and S. S. Shevkoplyas, *Lab Chip*, 2012, 12, 274-280.
8. W. R. Rodriguez, N. Christodoulides, P. N. Floriano, S. Graham, S. Mohanty, M. Dixon, M. Hsiang, T. Peter, S. Zavahir, I. Thior, D. Romanovicz, B. Bernard, A. P. Goodey, B. D. Walker and J. T. McDevitt, *PLoS Med.*, 2005, 2, e182.
9. H. M. Ji, V. Samper, Y. Chen, C. K. Heng, T. M. Lim and L. Yobas, *Biomed. Microdevices*, 2008, 10, 251-257.
10. U. Dharmasiri, M. A. Witek, A. A. Adams, J. K. Osiri, M. L. Hupert, T. S. Bianchi, D. L. Roelke and S. A. Soper, *Anal. Chem.*, 2010, 82, 2844-2849.
11. D. S. Reichmuth, S. K. Wang, L. M. Barrett, D. J. Throckmorton, W. Einfeld and A. K. Singh, *Lab Chip*, 2008, 8, 1319-1324.
12. P. Wilding, L. J. Kricka, J. Cheng, G. Hvichia, M. A. Shoffner and P. Fortina, *Anal. Biochem.*, 1998, 257, 95-100.
13. R. Carlson, C. V. Gabel, S. S. Chan, R. H. Austin, J. P. Brody and J. Winkleman, *Mol. Biol. Cell*, 1997, 8, 2407-2407.
14. H. Mohamed, L. D. McCurdy, D. H. Szarowski, S. Duva, J. N. Turner and M. Caggana, *IEEE Trans. Nanobioscience*, 2004, 3, 251-256.
15. S. J. Tan, L. Yobas, G. Y. Lee, C. N. Ong and C. T. Lim, *Biomed. Microdevices*, 2009, 11, 883-892.
16. D. Lee, P. Sukumar, A. Mahyuddin, M. Choolani and G. Xu, *J. Chromatogr. A*, 2010, 1217, 1862-1866.
17. X. Chen, D. Cui, C. Liu, H. Li and J. Chen, *Anal. Chim. Acta*, 2007, 584, 237-243.
18. P. Sethu, A. Sin and M. Toner, *Lab Chip*, 2006, 6, 83-89.
19. V. VanDelinder and A. Groisman, *Anal. Chem.*, 2007, 79, 2023-2030.
20. S. M. McFaul, B. K. Lin and H. Ma, *Lab Chip*, 2012, 12, 2369-2376.
21. S. Zheng, H. K. Lin, B. Lu, A. Williams, R. Datar, R. J. Cote and Y. C. Tai, *Biomed. Microdevices*, 2011, 13, 203-213.
22. L. R. Huang, E. C. Cox, R. H. Austin and J. C. Sturm, *Science*, 2004, 304, 987-990.
23. J. McGrath, M. Jimenez and H. Bridle, *Lab Chip*, 2014, 14, 4139-4158.
24. J. A. Davis, D. W. Inglis, K. J. Morton, D. A. Lawrence, L. R. Huang, S. Y. Chou, J. C. Sturm and R. H. Austin, *Proc. Natl. Acad. Sci. U. S. A.*, 2006, 103, 14779-14784.
25. K. Loutharback, J. D'Silva, L. Y. Liu, A. Wu, R. H. Austin and J. C. Sturm, *AIP Adv.*, 2012, 2, 042107.
26. D. W. Inglis, K. J. Morton, J. A. Davis, T. J. Zieziulewicz, D. A. Lawrence, R. H. Austin and J. C. Sturm, *Lab Chip*, 2008, 8, 925-931.

27. K. K. Zeming, T. Salafi, C. H. Chen and Y. Zhang, *Sci. Rep.*, 2016, 6, 22934.
28. K. Louthierback, K. S. Chou, J. Newman, J. Puchalla, R. H. Austin and J. C. Sturm, *Microfluid. Nanofluid.*, 2010, 9, 1143-1149.
29. M. Al-Fandi, M. Al-Rousan, M. A. K. Jaradat and L. Al-Ebbini, *Robot. Comput. Integr. Manuf.*, 2011, 27, 237-244.
30. D. R. Gossett, W. M. Weaver, A. J. Mach, S. C. Hur, H. T. K. Tse, W. Lee, H. Amini and D. Di Carlo, *Anal. Bioanal. Chem.*, 2010, 397, 3249-3267.
31. M. Yamada, M. Nakashima and M. Seki, *Anal. Chem.*, 2004, 76, 5465-5471.
32. J. Takagi, M. Yamada, M. Yasuda and M. Seki, *Lab Chip*, 2005, 5, 778-784.
33. M. Yamada and M. Seki, *Lab Chip*, 2005, 5, 1233-1239.
34. A. A. S. Bhagat, H. W. Hou, L. D. Li, C. T. Lim and J. Y. Han, *Lab Chip*, 2011, 11, 1870-1878.
35. Z. G. Wu, B. Willing, J. Bjerketorp, J. K. Jansson and K. Hjort, *Lab Chip*, 2009, 9, 1193-1199.
36. D. Di Carlo, *Lab Chip*, 2009, 9, 3038-3046.
37. H. Amini, W. Lee and D. Di Carlo, *Lab Chip*, 2014, 14, 2739-2761.
38. O. Otto, P. Rosendahl, A. Mietke, S. Golfier, C. Herold, D. Klaue, S. Girardo, S. Pagliara, A. Ekpenyong, A. Jacobi, M. Wobus, N. Topfner, U. F. Keyser, J. Mansfeld, E. Fischer-Friedrich and J. Guck, *Nat. Methods*, 2015, 12, 199-202.
39. S. C. Hur, N. K. Henderson-MacLennan, E. R. McCabe and D. Di Carlo, *Lab Chip*, 2011, 11, 912-920.
40. D. Di Carlo, J. F. Edd, D. Irimia, R. G. Tompkins and M. Toner, *Anal. Chem.*, 2008, 80, 2204-2211.
41. J. Sun, M. Li, C. Liu, Y. Zhang, D. Liu, W. Liu, G. Hu and X. Jiang, *Lab Chip*, 2012, 12, 3952-3960.
42. M. G. Lee, J. H. Shin, C. Y. Bae, S. Y. Choi and J. K. Park, *Anal. Chem.*, 2013, 85, 6213-6218.
43. P. R. Gascoyne and J. V. Vykoukal, *Proc. IEEE Inst. Electr. Electron. Eng.*, 2004, 92, 22-42.
44. P. Gascoyne, C. Mahidol, M. Ruchirawat, J. Satayavivad, P. Watcharasit and F. Becker, *Lab Chip*, 2002, 2, 70-75.
45. R. S. Thomas, H. Morgan and N. G. Green, *Lab Chip*, 2009, 9, 1534-1540.
46. S. Park, M. Koklu and A. Beskok, *Anal. Chem.*, 2009, 81, 2303-2310.
47. D. Lee, B. Hwang and B. Kim, *Micro Nano Syst. Lett.*, 2016, 4, 2-11.
48. J. Yang, Y. Huang, X. B. Wang, F. F. Becker and P. R. Gascoyne, *Biophys. J.*, 2000, 78, 2680-2689.
49. V. Gupta, I. Jafferji, M. Garza, V. O. Melnikova, D. K. Hasegawa, R. Pethig and D. W. Davis, *Biomicrofluidics*, 2012, 6, 024133.
50. J. Yang, Y. Huang, X. B. Wang, F. F. Becker and P. R. Gascoyne, *Anal. Chem.*, 1999, 71, 911-918.
51. D. J. O'Shannessy, D. W. Davis, K. Anderes and E. B. Somers, *Biomark Insights*, 2016, 11, 7-18.
52. N. Piacentini, G. Mernier, R. Tornay and P. Renaud, *Biomicrofluidics*, 2011, 5, 034122.
53. M. Yamashita, S. Miyata, S. Takeuchi and H. Inoue, *Conf. Proc. IEEE Eng. Med. Biol. Soc.*, 2013, 2013, 4462-4465.
54. S. Park, Y. Zhang, T. H. Wang and S. Yang, *Lab Chip*, 2011, 11, 2893-2900.
55. I. F. Cheng, C. C. Lin, D. Y. Lin and H. C. Chang, *Biomicrofluidics*, 2010, 4, 034104.
56. A. Valero, T. Braschler, N. Demierre and P. Renaud, *Biomicrofluidics*, 2010, 4, 022807.
57. J. Voldman, M. L. Gray, M. Toner and M. A. Schmidt, *Anal. Chem.*, 2002, 74, 3984-3990.
58. J. Suehiro, D. Noutomi, M. Shutou and M. Hara, *J. Electrostat.*, 2003, 58, 229-246.

59. F. F. Becker, X. B. Wang, Y. Huang, R. Pethig, J. Vykoukal and P. R. Gascoyne, *Proc. Natl. Acad. Sci. U. S. A.*, 1995, 92, 860-864.
60. R. Burger, D. Kirby, M. Glynn, C. Nwankire, M. O'Sullivan, J. Siegrist, D. Kinahan, G. Aguirre, G. Kijanka, R. A. Gorkin, 3rd and J. Ducree, *Curr. Opin. Chem. Biol.*, 2012, 16, 409-414.
61. L. Riegger, M. Grumann, J. Steigert, S. Lutz, C. P. Steinert, C. Mueller, J. Viertel, O. Prucker, J. Ruhe, R. Zengerle and J. Ducree, *Biomed. Microdevices*, 2007, 9, 795-799.
62. J. Zhang, Q. Guo, M. Liu and J. Yang, *J. Micromech. Microeng.*, 2008, 18, 125025-125030.
63. S. Haeberle, T. Brenner, R. Zengerle and J. Ducree, *Lab Chip*, 2006, 6, 776-781.
64. D. J. Kinahan, S. M. Kearney, M. T. Glynn and J. Ducree, *Sens. Actuators, A*, 2014, 215, 71-76.
65. S. T. Moen, C. L. Hatcher and A. K. Singh, *PLoS One*, 2016, 11, e0153137.
66. H. Shiono, S. Ogawa, T. Matsui, S. Niwata, T. Okada and Y Ito, *J. Physiol. Sci.*, 2010, 60, S173.
67. Unitaid,
http://unitaid.org/images/marketdynamics/publications/UNITAID_HIV_Nov_2015_Dx_Landscape.PDF
68. AlereHIV,
<http://www.alerehiv.com/content/alerehiv/ww/home/hiv-monitoring/alere-pima-CD4/introduction.html>
69. BDBioscience, <https://www.bdbiosciences.com/documents/FACSPresto-BR.pdf>
70. M. Beck, S. Brockhuis, N. van der Velde, C. Breukers, J. Greve and L. W. M. M. Terstappen, *Lab Chip*, 2012, 12, 167-173.
71. X. Zhang, D. Wasserberg, C. Breukers, L. W. M. M. Terstappen and M. Beck, *Analyst*, 2016, 141, 3068-3076.
72. X. Zhang, D. Wasserberg, C. Breukers, L. W. M. M. Terstappen and M. Beck, *ACS Appl. Mater. Interfaces*, 2016, 8, 27539-27545.
73. S. Jin, Dai M., B Ye and S. R. Nugen, *Microsyst. Technol.*, 2013, 19, 2011-2015.
74. M. Mortato, L. Blasi, G. Barbarella, S. Argentiore and G. Gigli, *Biomicrofluidics*, 2012, 6, 044107.
75. I. Safarik and M. Safarikova, *J. Chromatogr. B*, 1999, 722, 33-53.
76. V. I. Furdui and D. J. Harrison, *Lab Chip*, 2004, 4, 614-618.
77. K. Hoshino, Y. Y. Huang, N. Lane, M. Huebschman, J. W. Uhr, E. P. Frenkel and X. Zhang, *Lab Chip*, 2011, 11, 3449-3457.
78. D. W. Inglis, R. Riehn and P. Austin, *Appl. Phys. Lett.*, 2004, 85, 5093-5095.
79. A. E. Saliba, L. Saias, E. Psychari, N. Minc, D. Simon, F. C. Bidard, C. Mathiot, J. Y. Pierga, V. Fraissier, J. Salamero, V. Saada, F. Farace, P. Vielh, L. Malaquin and J. L. Viovy, *Proc. Natl. Acad. Sci. U. S. A.*, 2010, 107, 14524-14529.
80. D. Horak, Z. Svobodova, J. Autebert, B. Coudert, Z. Plichta, K. Kralovec, Z. Bilkova and J. L. Viovy, *J. Biomed. Mater. Res. A*, 2013, 101, 23-32.
81. F. Paul, S. Roath, D. Melville, D. C. Warhurst and J. O. Osisanya, *Lancet*, 1981, 2, 70-71.
82. T. F. Didar and M. Tabrizian, *Lab Chip*, 2010, 10, 3043-3053.
83. S. Nagrath, L. V. Sequist, S. Maheswaran, D. W. Bell, D. Irimia, L. Ulkus, M. R. Smith, E. L. Kwak, S. Digumarthy, A. Muzikansky, P. Ryan, U. J. Balis, R. G. Tompkins, D. A. Haber and M. Toner, *Nature*, 2007, 450, 1235-1239.

84. B. D. Plouffe, T. Kniazeva, J. E. Mayer, S. K. Murthy and V. L. Sales, *FASEB J.*, 2009, 23, 3309-3314.
85. Z. Wang, S. Y. Chin, C. D. Chin, J. Sarik, M. Harper, J. Justman and S. K. Sia, *Anal. Chem.*, 2010, 82, 36-40.
86. X. Cheng, D. Irimia, M. Dixon, K. Sekine, U. Demirci, L. Zamir, R. G. Tompkins, W. Rodriguez and M. Toner, *Lab Chip*, 2007, 7, 170-178.
87. X. Cheng, A. Gupta, C. Chen, R. G. Tompkins, W. Rodriguez and M. Toner, *Lab Chip*, 2009, 9, 1357-1364.
88. N. N. Watkins, U. Hassan, G. Damhorst, H. Ni, A. Vaid, W. Rodriguez and R. Bashir, *Sci. Transl. Med.*, 2013, 5, 214-224.
89. B. D. Plouffe, M. A. Brown, R. K. Iyer, M. Radisic and S. K. Murthy, *Lab Chip*, 2009, 9, 1507-1510.
90. X. Guan, H. J. Zhang, Y. N. Bi, L. Zhang and D. L. Hao, *Biomed. Microdevices*, 2010, 12, 683-691.
91. H. Ulrich, J. E. Ippolito, O. R. Pagan, V. A. Eterovic, R. M. Hann, H. Shi, J. T. Lis, M. E. Eldefrawi and G. P. Hess, *Proc. Natl. Acad. Sci. U. S. A.*, 1998, 95, 14051-14056.
92. Q. Chen, J. Wu, Y. Zhang, Z. Lin and J. M. Lin, *Lab Chip*, 2012, 12, 5180-5185.
93. J. A. Phillips, Y. Xu, Z. Xia, Z. H. Fan and W. Tan, *Anal. Chem.*, 2009, 81, 1033-1039.
94. Y. Xu, J. A. Phillips, J. Yan, Q. Li, Z. H. Fan and W. Tan, *Anal. Chem.*, 2009, 81, 7436-7442.
95. W. Liu, H. Wei, Z. Lin, S. Mao and J. M. Lin, *Biosens. Bioelectron.*, 2011, 28, 438-442.

Chapter 2

Real-time *in situ* fluorescence imaging system for the measurement of on-chip antibody release

Xichen Zhang, Christian Breukers, Leon W. M. M. Terstappen, Markus Beck

Abstract

Here we develop a novel method to study the release of fluorophore-labeled antibody from a hydrogel matrix in a microdevice by real-time in situ fluorescence imaging. The understanding of this on-chip release provides fruitful insights to realize on-chip sample preparation in a simple manner for a variety of biochemical applications, in particular immunostaining based cell counting which is introduced in the following chapters. In this chapter, the detailed information of this method including sample characterization, system establishment, signal validation and quantification are described.

2.1 Introduction

On-chip sample preparation integrated in microdevices has many advantages for point-of-Care (POC) diagnostics.¹ The small dimension of microfluidic systems makes turbulent mixing difficult. Therefore, common concepts in microfluidics to achieve the desired mixing of integrated reagents and sample fluids often employ sophisticated fluid actuation and precisely flow control.² However, the complexity of instrumentation and operating procedures discourages this concept to be used for applications in low-resource settings.

Recently, the incorporation of hydrogel materials in microdevices to embed and release reagents receives more and more attention in the fields of tissue engineering and in-vitro diagnostics.³⁻⁵ Hydrogels are good matrices to store reagents and capable of releasing reagents in a controlled manner based on their physiochemical properties and their interaction with embedded reagents, thus having the potential to achieve desired sample-reagent mixing. Compared with the fabrication of complex microstructures, the concept of depositing hydrogel materials in simply designed microdevices is much easier and cheaper, making it well-suited for low-resource settings.

Our cell counting chamber is one of the first concepts to achieve on-chip controlled reagent release for passive and diffusive mixing with the sample using a simple design.⁶ The counting chamber contains a gelatin layer with embedded antibody to enable on-chip sample preparation for the enumeration of CD4 positive (CD4⁺) T-lymphocytes. The delayed antibody release from the gelatin layer after rehydration by sample inflow ensures homogeneous mixing between blood and antibody, thus allowing for uniform cell staining. To fully explore the potential of controlled reagent release from hydrogels for on-chip sample preparation in microdevices, the characterization of on-chip release kinetics and the understanding of the underlying mechanisms are essential.

However, the release process in microfluidic systems is so far not addressed in drug delivery research. Conventional controlled release studies aim for sustained drug release, on a timescale from hours to days, to prevent the need to give the drugs at multiple time points.⁷ Yet, a rapid diagnostic test requires the completion of sample

preparation within a few minutes, which is a much shorter timespan for reagent release. To gain a detailed understanding of on-chip release processes, a proper method for the characterization and quantification of the release process is required. Often, especially in drug delivery studies, this is done by quantifying the concentration of the released substance in an eluting medium at hour-scale intervals. However, this method is not applicable to monitor on-chip release, because repetitive collection of minute volumes (μL) of the medium from a microdevice at short intervals (seconds) and numerous measurements of the low reagent concentrations within small sample volumes are time-consuming and challenging.

Herein, we employed a novel method to determine the release kinetics by quantifying the antibody remaining in a gelatin layer during the release process. First of all, a gelatin layer containing fluorophore-labeled antibody was deposited on a substrate slide within a defined region around which a frame of flow chamber was created. Then, an eluting medium was pumped through the chamber at a controlled rate to wash away the released antibody. Meanwhile, a fluorescence imaging system was used to measure the intensity of the gelatin/antibody layer in real-time and *in situ* manner. The detected fluorescence signal was corrected by taking temperature-dependent excitation output and photobleaching into account. After calibration, the detected intensity was proven to be a measure of antibody quantity. Hence, the reduction rate of layer intensity over time reflects the kinetics of antibody release.

Thanks to this powerful method, the release profiles of antibody from differently prepared gelatin layers and other hydrogel layers are obtained and used to facilitate on-chip sample preparation for immunostaining based cell counting in the following chapters. This valuable information not only uncovers the theory of the underlying release mechanism but also provides practical guidance to pursue on-chip sample preparation with controlled reagent release for a variety of applications in POC diagnostics.

2.2 Flow chamber fabrication and layer preparation

An assembled flow chamber is shown schematically in Fig. 2.1a and 2.1b. Precut laminating adhesive (nominal thickness 0.001 inch = 25.4 μm , 3M) was attached to a poly(methyl methacrylate) (PMMA) substrate (76 mm $\times$ 26 mm $\times$ 1 mm) to create a channel (61 mm $\times$ 4.8 mm). The following attachment of poly(tetrafluoroethylene) (PTFE) tapes determines an area (4.8 mm $\times$ 4 mm) to deposit gelatin (type A, bloom 295 g, Sigma-Aldrich)/allophycocyanin labeled antiCD3 IgG (APC- α CD3, clone SK7, ~260 kDa, BD, referring to Becton Dickinson for all the following chapters) solution. Two different deposition methods, manual casting and inkjet printing, are described in this thesis. The details of the casting method are covered in Chapter 3 and 6; while in Chapter 4, 5 and 7, inkjet printing technology is employed to deposit the layers. In this chapter, only cast layers are used to demonstrate the release measurement. After the solution was dried, the PTFE tape was removed prior to the final attachment of a capping glass slide (Menzel, 76 mm $\times$ 26 mm $\times$ 1 mm) with two holes to connect the tubing. The tubing was connected to the channel through Nanoport™ Assemblies (Upchurch Scientific). Fig. 2.1c is a camera image of an assembled flow chamber.

To prepare the casting solution, gelatin powder was dissolved in milliQ water at 37°C for 1 h while stirring. Gelatin solution and APC- α CD3 stock solution were both centrifuged at 21000 $\times$ g for 10 min at 37°C to collect the supernatant before use. 13.5 μL of 0.2% m/v gelatin solution containing 1.5 $\mu\text{g mL}^{-1}$ APC- α CD3 was cast in the defined area and dried at 20°C in ambient atmosphere to obtain a gelatin/APC- α CD3 layer. After the layer was dried, material of a small area was scraped off the layer to expose the substrate, which was used as a reference surface for the characterization of layer thickness.

Fig. 2.1: Schematic representations of flow chambers a) Perspective view, b) Side view. c) Camera image of an assembled flow chamber.

2.3 Fluorescence imaging system

A custom-built fluorescence imaging system (Fig. 2.2a) was employed to measure the fluorescence of the layer. The imaging system contains two LEDs: one red (625 ± 10 nm) and one blue (460 ± 10 nm) from the same manufacturer (PhlatLight CBT-40, Luminus Devices). In this chapter, we focus on demonstrating the method to monitor the release of APC- α CD3 using red excitation. Excitation light from the red LED passes through a 650 nm short pass filter (Semrock) prior to exposing to the sample. The emitted fluorescence passes through a 685/40 nm band pass filter (Semrock) and is imaged at $1.7\times$ magnification by a CCD camera (SBIG). The focal depth of the imaging system is ~ 0.2 mm. More details of the imaging setup have been described previously.⁶ With respect to imaging peridinin-chlorophyll labeled antiCD4 IgG (PerCP- α CD4) labeled cells, the blue LED was enabled. The emitted fluorescence from PerCP- α CD4 can be acquired in the same way without changing the setup of the imaging system (using the same emission filter and lens).

The fluorescence image of the layer in the chamber is shown in Fig. 2.2b. The increased fluorescence observed at the sides of the layer is mainly attributed to an accumulation of APC- α CD3 due to the flux of APC- α CD3 towards layer boundary during drying.

Fig. 2.2: a) Schematic representations of the fluorescence imaging setup⁶. b) Fluorescence image of the gelatin/APC- α CD3 layer. Scale bar 0.5 mm.

2.4 Characterization of layer thickness

The topography of the gelatin layer was measured using a white light interferometer (smartWLI-microscope, GBS) with approximately 2 μm lateral resolution and 10 nm vertical resolution. The measurement principle is explained in detail by Kim and coworkers.⁸ Briefly, a light beam from a white light source is split in a Mirau objective (10 $\times$, Nikon) into a measurement beam and a reference beam. The measurement beam reflects off the surface of the sample (gelatin layer) and the reference beam is reflected by a reference mirror located in the objective. The two reflected beams are superimposed and detected by a microscope camera, generating a sample image with interference patterns. When the optical paths of the two beams are equal in length for a given point at the layer surface, there is a constructive interference for all wavelengths in the spectrum of the white light, thus resulting in a maximum intensity at the respective camera pixel. The difference between the reference beam and the measurement beam is regulated by vertically adjusting the height of the objective above the layer (z-height) by a built-in piezo actuator. To match the length of the reference beam for every single point at the layer surface, the objective scans stepwise within a defined range (10 μm) and the camera acquires a stack of sample images with varying interference patterns, which contain the digitized intensity for each pixel. The interferograms containing the height-dependent intensity information for each pixel are analyzed (fitted) by the supplied software to determine the height of the sample at every position. Three positions of the exposed substrate surface are used as the reference height ($z = 0$) to correct for the tilt placement of the sample. Thus, the layer topography is obtained.

Fig. 2.3a shows the topography of the layer across the channel. The zoomed-in fluorescence image of the same region (Fig. 2.3b) shows an almost identical pattern, indicating that no significant de-mixing of gelatin and APC- αCD3 happens during the casting process. As shown in Fig. 2.3c, a smooth and flat layer with a thickness of approximately 250 nm can be obtained in the central region.

Fig. 2.3: a) Topography of a segment of a gelatin layer across the channel with a cross scratch. Scale bar 0.5 mm. b) Zoom-in fluorescence image showing the same segment region. c) Thickness profile along the pink line in a).

2.5 Characterization of chamber height

The chamber height was measured by a custom-built laser interferometer. (manuscript in preparation) Briefly, the light from an edge-emitting laser diode ($\lambda = 405$ nm, beam divergence FWHM $\sim 20^\circ$) is collimated and focused at a single point of a flow chamber with two high numerical aperture lenses (diameter = focal length = 25.4 mm). The transmitted light is collimated by another lens of the same type, filtered by neutral density filters and falls onto the CCD chip of a camera. Since part of the light is reflected on the inner surfaces of the chamber, the resulting path length difference between the beams leads to constructive (path difference = $n \times \lambda$) or destructive (path difference = $(n + 1/2) \times \lambda$) interference, depending on the transmission angle (θ),

which corresponds to different positions on the camera. The resulting interferogram is analyzed by plotting the intensity against $\cos(\theta)$, resulting in a periodic intensity profile, the periodicity of which, determined by Fourier analysis, yields the chamber height.

For each chamber, three points within the channel region were randomly selected for the measurement. The chamber height was obtained as a mean of the three measurements. Nine chambers fabricated on different days were measured to check the reproducibility in height. The obtained height is $26.6 \pm 0.4 \mu\text{m}$ ($n = 9$).

2.6 Correction of measured fluorescence intensity

In the fluorescence imaging system, continuous illumination generates substantial amount of heat and raises the temperature of the LED chip, which can result in attenuated excitation output. Therefore it is of great importance to calibrate the compromised performance of the LED with temperature and correct the measured fluorescence intensity of the layer accordingly. The temperature was monitored by a built-in Pt thermistor (NTC, Murata Manufacturing) on the copper back plate of the LED chip. The actual temperature value was derived from the reading of the thermistor. The excitation output was correlated to the intensity of scattered light from a rough tissue paper during the illumination. The intensity at a given temperature ($I_{\text{scatter @ } T}$) was measured by a built-in detector. To determine the influence of the temperature on the excitation output of the LED, the relative luminous flux at a given temperature (the ratio of $I_{\text{scatter @ } T}$ against $I_{\text{scatter @ } 21^\circ\text{C}}$, where the output at 21°C was regarded as 100% luminous flux) was plotted against the corresponding temperature. In Fig. 2.4, it is clearly shown that the excitation output, which is characterized by the relative luminous flux deteriorates proportionally against the temperature. As the fluorescence intensity of emission is proportional to the excitation intensity, the measured fluorescence intensity of the gelatin/APC- αCD3 layer can be corrected accordingly to obtain the actual intensity.

Fig. 2.4: Decline of the excitation output with increasing LED temperature.

2.7 Calibration of fluorescence intensity against the quantity of APC- α CD3

The integrated fluorescence intensity from a region of interest (ROI) in the image was used as a measure to quantify the amount of deposited antibody. To validate this quantification, the calibration of integrated intensity against the quantity of APC- α CD3 in both dry state (in the dry layer) and wet state (in phosphate buffered saline (PBS)) were performed in the flow chamber. Dry gelatin layers were prepared by applying the same casting protocol as described before. (A 13.5 μ L of 0.2% w/v gelatin solution with varying APC- α CD3 concentration ranging from 0.18 μ g mL⁻¹ to 1.5 μ g mL⁻¹ was deposited in an area of 4.8 mm $\times$ 4 mm.) The integrated fluorescence intensity of the layer in the ROI (4.8 mm $\times$ 4 mm) was obtained after the subtraction of autofluorescence and the correction of temperature-dependent excitation output. The integrated intensity in the ROI was plotted against the calculated APC- α CD3 quantity in the layer. (Fig. 2.5a) In comparison, PBS with various APC- α CD3 concentrations (0.18 μ g mL⁻¹ to 1.5 μ g mL⁻¹) was filled into the flow chamber. A ROI with the same size in area (approximately 0.51 μ L solution) was imaged and the integrated intensity was plotted against the calculated quantity of APC- α CD3. (Fig. 2.5b) The linear relationship in both Fig. 2.5a and 2.5b demonstrates the validity of integrated intensity to quantify

APC- α CD3. In dry layers, we detected 0.43 camera units per second (CPS) per APC- α CD3 molecule. Likewise in wet condition, we detected 0.45 camera units per second (CPS) per APC- α CD3 molecule.

Fig. 2.5: Calibrations of integrated intensity against the quantity of APC- α CD3 in dry gelatin a) and in PBS buffer b). Data points represent mean $\pm$ standard deviation ($n = 3$).

2.8 Measurement of release kinetics

PBS was used as the eluting medium in the release experiments. Upon the filling of PBS into the flow chamber, the gelatin layer is hydrated and the embedded APC- α CD3 becomes mobile to diffuse into PBS. Unlike conventional drug delivery studies, which quantify the release reagent in the eluting medium, our method quantifies the remaining APC- α CD3 in the layer over time to determine the release kinetics. To achieve this, a real-time and *in situ* release measurement was carried out; in which time-lapse fluorescence imaging and dynamic flow condition are employed.

With respect to time-lapse imaging, the red LED was programmed in LabVIEW (National Instrument) to illuminate the layer for 1 s with intervals of 5 s. During the illumination, the fluorescence image of the layer was acquired by the camera. To test the bleaching of APC- α CD3 in both dry and wet state, an unfilled chamber with a coated gelatin/APC- α CD3 layer and a filled chamber with PBS/APC- α CD3 solution (static condition) were subject to the illumination cycle for 10 min (release period). The developments of the integrated intensity in both chambers are shown in Fig. 2.6. The fluorescence of APC- α CD3 in both states shows insignificant difference in intensity over 10 min, suggesting negligible bleaching during the release process. Therefore, the measured intensity does not require further correction.

Fig. 2.6: Development of fluorescence intensity of APC- α CD3 in different states during time-lapse imaging. Data points (shown with 20 s intervals to prevent overcrowded plots) represent mean $\pm$ standard deviation ($n = 3$).

The focal depth of the lens (~ 0.2 mm) is orders of magnitude greater than the layer thickness and still considerably larger than the chamber height. As a result, both APC- α CD3 embedded in the gelatin layer and released in PBS will be focused, thus reducing the amount of out-of-focus fluorescence in the background and achieving accurate and precise quantification of fluorescence intensity values.⁹

To solely monitor the fluorescence of the layer, PBS has to be refreshed in a timely manner to maintain the concentration of APC- α CD3 at a negligible level. PBS was regulated to flow through the chamber in a controlled rate by a syringe pump. Insufficient flow rate might underestimate the release due to the presence of released APC- α CD3 located on top of the layer. A minimum flow rate of $\sim 30 \mu\text{L min}^{-1}$ (a threshold rate), which can refresh a volume ($4.8 \text{ mm} \times 4 \text{ mm} \times \sim 26 \mu\text{m} = \sim 0.5 \mu\text{L}$) of PBS filling the space on top of the layer in the flow chamber within 1 s (exposure time), is required to ensure the accuracy of the measurement.

Before the filling of PBS, a ROI of $0.5 \text{ mm} \times 0.5 \text{ mm}$ in the central region of the layer was defined. A thickness of 250 nm was determined. The integrated intensities of this ROI from all time-lapse images captured during the release were measured. After the subtraction of autofluorescence and the correction of temperature-dependent

excitation output, the remaining APC- α CD3 in the layer during the release process was determined. Thanks to the consistent average fluorescence yield per molecule in both dry and wet states, the intensity of the dry layer was used as a reference defining the total amount of APC- α CD3 (M_{total}) in the layer. The ratio of intensity reduction at a given time point over the reference intensity (M_t/M_{total}) was used to determine the fractional release. The release profiles of APC- α CD3 from 250 nm thick gelatin layers subject to PBS flow at different flow rates are shown in Fig. 2.7. The identical release profiles confirm that all the flow rates (greater than the threshold rate) are sufficient to maintain a perfect sink condition for antibody release, in which the concentration gradient of APC- α CD3 between the layer and PBS is always maximal. In addition, the flow rate-independent release kinetics suggests that perfusion plays a minor role in the release and proves that the release takes place at the interface between the layer surface and PBS. In the end, 60 $\mu\text{L min}^{-1}$ was chosen as a standard flow rate as it mimics the blood inflow driven by capillary force in cell counting applications discussed in the following chapters.

Fig. 2.7: Influence of flow rate on the release of APC- α CD3 from 250 nm thick gelatin layers.

2.9 Conclusion

A novel method to monitor on-chip antibody release is demonstrated in this chapter. The introduction of dynamic flow conditions and a custom-built fluorescence imaging system enables a real-time and *in situ* measurement of release kinetics from a gelatin layer. This method is a powerful tool to study on-chip reagent release and the discovery of desired release kinetics will be beneficial for the development of miniaturized biochemical assays with on-chip sample preparation.

References

1. M. Hitzbleck and E. Delamarche, *Chem. Soc. Rev.*, 2013, 42, 8494-8516.
2. F. Cui, M. Rhee, A. Singh and A. Tripathi, *Annu. Rev. Biomed. Eng.*, 2015, 17, 267-286.
3. S. Cosson and M. P. Lutolf, *Sci. Rep.*, 2014, 4, 4462.
4. D. Puchberger-Enengl, C. Krutzler, F. Keplinger and M. J. Vellekoop, *Lab Chip*, 2014, 14, 378-383.
5. R. R. Niedl and C. Beta, *Lab Chip*, 2015, 15, 2452-2459.
6. M. Beck, S. Brockhuis, N. van der Velde, C. Breukers, J. Greve and L. W. M. M. Terstappen, *Lab Chip*, 2012, 12, 167-173.
7. T. R. Hoare and D. S. Kohane, *Polymer*, 2008, 49, 1993-2007.
8. S. W. Kim and G. H. Kim, *Appl. Opt.*, 1999, 38, 5968-5973.
9. J. C. Waters, *J. Cell Biol.*, 2009, 185, 1135-1148.

Chapter 3

Controlled antibody release from gelatin for on-chip sample preparation

Xichen Zhang, Dorothee Wasserberg, Christian Breukers, Leon W. M. M. Terstappen, Markus Beck

This chapter has been published as:
Analyst, 2016, 141, 3068-3076

Abstract

A practical way to realize on-chip sample preparation for point-of-care diagnostics is to store the required reagents on a microfluidic device and release them in a controlled manner upon contact with the sample. For the development of such diagnostic devices, a fundamental understanding of the release kinetics of reagents from suitable materials in microfluidic chips is therefore essential. Here, we study the release kinetics of fluorophore-labeled antibodies from (sub-) μm thick gelatin layers and several ways to control the release time. The observed antibody release is well-described by a diffusion model. Release times ranging from ~ 20 s to ~ 650 s were determined for layers with thicknesses (in the dry state) between $0.25\ \mu\text{m}$ and $1.5\ \mu\text{m}$, corresponding to a diffusivity of $0.65\ \mu\text{m}^2\ \text{s}^{-1}$ (in the swollen state) for our standard layer preparation conditions. By modifying the preparation conditions, we can influence the properties of gelatin to realize faster or slower release. Faster drying at increased temperatures leads to shorter release times, whereas slower drying at increased humidity yields slower release. As expected in a diffusive process, the release time increases with the size of the antibody. Moreover, the ionic strength of the release medium has a significant impact on the release kinetics. Applying these findings to cell counting chambers with on-chip sample preparation, we can tune the release to control the antibody distribution after inflow of blood in order to achieve homogeneous cell staining.

3.1 Introduction

One of the major fields of applications for microfluidics is *in vitro* diagnostics, with great potential particularly in point-of-care diagnostics.¹⁻³ Many process steps and sensing principles have been realized on microfluidic devices.⁴ Recently, on-chip sample preparation using reagents stored in microfluidic devices has received more and more attention.⁵ On-chip sample preparation can eliminate the dependence on external instrumentation for reagent delivery as well as benchtop sample treatment, thus integrating the complete test into one disposable.⁶ To realize on-chip sample preparation, reagents are integrated in microfluidic devices and released upon contact with the inflowing sample. Compared with liquid reagents⁷⁻¹⁰, integrating dry reagents in microfluidic devices is beneficial for long-term storage and convenient for transportation, due to the better stability of reagents in the dry state.¹¹ However, the controlled dissolution of the reagent and on-chip mixing with the added sample is challenging. Especially in applications where the inflowing sample passes a reservoir of the reagent and slowly dissolves or washes out the stored reagent¹²⁻¹⁵, very precise control of the sample flow and the reagent release process is required. In contrast, mixing between sample and reagents in stopped flow applications can be realized simply by releasing the reagent with sufficient delay after the sample inflow has stopped at the required position on the chip.¹⁶ To realize controlled reagent release, hydrogels with embedded reagents in microfluidics chambers have been employed in the past. It has been shown that sample inflow can induce swelling due to rehydration¹⁷ or a change in pH¹⁸ and lead to subsequent diffusion of reagents out of the hydrogel matrix.

We previously demonstrated this principle for on-chip sample preparation in cell counting chambers for the enumeration of CD4 positive (CD4⁺) T-lymphocytes in a stopped-flow configuration.¹⁷ In short, our cell counting chambers contain a dry gelatin layer with embedded fluorophore-labeled antibody for the immunostaining of T-lymphocytes. Inflowing blood initiates the rehydration (i.e. swelling) of the gelatin matrix but release only starts after a certain level of rehydration has been reached. The layers are tailored in such a way, that the inflow of blood takes place, while rehydration is not yet sufficient for antibody release. However, after the chamber is

filled and the flow has stopped, rehydration rapidly reaches levels that enable release. The delayed release after inflow (into a static sample) ensures, that wash-off of antibody during inflow is largely prevented, and that the released antibody is distributed homogeneously throughout the chamber. Homogeneous antibody release is a prerequisite for uniform cell staining, which is of paramount importance to our cell counting application: In our imaging setup we chose a wide field of view at low magnification in order to image a large volume at once, and can therefore identify stained cells only via their fluorescence intensity. If the staining is inhomogeneous the definition of selection criteria like an intensity threshold becomes problematic.

To further optimize the delayed release, a fundamental understanding of the release kinetics is essential. Conventional release studies generally focus on long-term release within hours or days from polymer layers tens of μm to mm thick for the purpose of *in vivo* drug delivery.¹⁹ Microfluidics applications generally require thinner layers and shorter release times. Our cell counting application, for instance, requires sub- μm gelatin layers to release fluorophore-labeled antibody on timescales between 10 seconds and a few minutes. This range is pre-determined by the time needed to fill the chamber by capillary action (between 2 s and 10 s, representing the lower limit for the release) and the incubation time for immunostaining (typically 5 min to 30 min, during which sufficient antibody has to be released).

Here, we report methods to monitor and influence the release of antibodies from gelatin in flow chambers. In our release experiments, a medium was passed through a flow chamber coated on one side with a dry gelatin layer containing fluorophore-labeled antibody, and the decrease of fluorescence intensity during release was monitored over time. The measured release kinetics was found to be consistent with a diffusion-controlled release model. Various parameters including layer thickness, drying conditions (temperature and humidity), antibody size and the ionic strength of the medium allowed us to tune the release kinetics. Moreover, we verified that the release kinetics determine the antibody distribution as well as the homogeneity of cell staining in a counting chamber after sample inflow.

3.2 Experiments

3.2.1 Flow chamber and cell counting chamber fabrication

The assembled flow chamber is shown schematically in Fig. 3.1a and 3.1b. Precut laminating adhesive (nominal thickness 25.4 μm , 3M) was attached to a poly(methyl methacrylate) (PMMA) substrate (76 mm $\times$ 26 mm $\times$ 1 mm) to create the flow channel (61 mm $\times$ 4.8 mm). The subsequent attachment of poly(tetrafluoroethylene) (PTFE) tape determined the casting area (4.8 mm $\times$ 4 mm) for the gelatin (type A, bloom 295 g, Sigma-Aldrich)/antibody-conjugate solution. The solution was left to dry to form a layer of typically a few hundred nm thickness. The PTFE tape was removed prior to the attachment of a capping glass slide (Menzel, 76 mm $\times$ 26 mm $\times$ 1 mm) with two holes for tubing connection. Actual chamber heights around 27 μm were determined interferometrically. Details on the reproducibility of device fabrication can be found in the Supporting Information (SI) section “Reproducibility of the gelatin layers prepared using ‘standard conditions’ and chamber heights”. The fabrication of cell counting chambers (Fig. 3.1c and 3.1d) is described in detail elsewhere.¹⁷ Briefly, the gelatin/antibody-conjugate layer was cast in a chamber (15 mm $\times$ 9 mm) created by cut-outs in laminating adhesive on a substrate slide. The chamber was covered by a capping slide with openings on both ends.

Fig. 3.1: Schematic representations of flow chambers a) Perspective view, b) Side view and cell counting chambers c) Perspective view, d) Side view.

3.2.2 Layer preparation

To determine the influence of several parameters on the release kinetics, we define “standard conditions” to prepare gelatin/antibody-conjugate layers and investigate variations thereof. Under “standard conditions”, gelatin powder was dissolved in milliQ water at 37°C for 1 h while stirring. The solution was centrifuged at 21000×g for 10 min at 37°C and the supernatant was used for all further experiments. A casting solution containing 0.2% w/v gelatin and 1.5 $\mu\text{g mL}^{-1}$ allophycocyanin labeled antiCD3 IgG (IgG, clone SK7, ~260 kDa, BD) was prepared. 20 μL of this solution was cast onto the flow chamber. The solution was dried at 20°C in ambient atmosphere with ~40% relative humidity (RH) to obtain a layer with a thickness of ~0.5 μm in the central region. By varying the volume of the same casting solution, we obtained layers of various thicknesses and determined the influence of the layer thickness on the release time. As a control, pure IgG solution without addition of gelatin was cast on a substrate slide. Since the pure IgG solution also contains trace amounts of gelatin as stabilizing agent, the thickness of this control layer is estimated to be 6 nm.

The effect of increased temperature (35°C and 40°C), increased humidity (85% RH) or reduced pressure (*in vacuo*) during the drying process on the release kinetics was determined.

3.2.3 Released molecules

The influence of the antibody size on the release kinetics, was studied by replacing IgG with a larger antibody, allophycocyanin labeled IgM (IgM, ~1495 kDa, Ebioscience) and with smaller antibody fragments labeled with smaller fluorophores: F(ab')₂-Alexa Fluor®647 (Fab, ~110 kDa, Cell Signaling) and Fc-Dylight®650 (Fc, ~50 kDa, Abcam).

3.2.4 Fluorescence imaging

A custom-built fluorescence imaging system was used to measure the release kinetics. Light from a red LED (625 ± 10 nm, CBT-40, Luminus Devices), passing through a 650 nm short pass filter (Semrock), is used to excite the fluorophores in the sample. The emitted fluorescence passes through a 685/40 nm band pass filter (Semrock) and is imaged at 1.7× magnification by a CCD camera (SBIG). Details of the imaging setup have been described previously.¹⁷

3.2.5 Layer characterization

The topography of gelatin/antibody-conjugate layers was determined using a white light interferometer (smartWLI-microscope, GBS) with about 10 nm resolution. Scraping off a small area in the central region of the gelatin layer allowed us to use the substrate as a reference. Fig. 3.2a shows the thickness distribution of a representative gelatin/IgG layer with smooth surface. The similar pattern of antibody distribution (fluorescence readout) shown in Fig. 3.2b indicates no significant de-mixing of gelatin and IgG in the dry layer. To establish a correlation between thickness and fluorescence of gelatin/IgG layers, a region of interest (ROI) of 0.5 mm $\times$ 0.5 mm located adjacent to the scratch was defined. The average thickness and the fluorescence intensity of ROIs in layers prepared under different conditions were measured. As shown in Fig. 3.3, the average thickness of a ROI is proportional to its fluorescence intensity.

Fig. 3.2: a) Contour plot. b) Fluorescence image of a dry gelatin/IgG layer.

Fig. 3.3: Calibration of the fluorescence intensity of the layer against the layer thickness. Data points represent mean $\pm$ standard deviation ($n = 3$).

3.2.6 Measurement of equilibrium mass swelling ratios

To determine the equilibrium mass swelling ratios of gelatin layers prepared under different conditions, three Petri dishes ($\varnothing$: 3 cm) were filled with 5 ml of 0.2% w/v gelatin solution each and dried under different conditions: 40% RH 20°C, 40% RH 40°C and 85% RH 20°C. After drying, gelatin layers of ~ 10 mg were obtained. 5 ml of PBS was added to each Petri dish to incubate the layer at 20°C overnight. No further weight increase was found for longer incubation times. Excess water from the surface was removed by filter paper before the swollen layers were weighed. After that, the swollen layers were dried again, and their weights did not differ from the initial values, suggesting negligible dissolution and loss of gelatin during incubation and excess water removal. The equilibrium mass swelling ratio was calculated by dividing the weight of the swollen layer by the weight of the initial layer. As the swelling is a one dimensional expansion in the experiments, the thickness of the swollen layer can be calculated from the thickness of the dry layer and the equilibrium mass swelling ratio, when assuming the density of gelatin to be 1.3 g cm^{-3} .²⁰

3.2.7 Measurement of release kinetics in flow chambers

Phosphate buffered saline (PBS) was used as the standard medium to flow at a rate of $1 \mu\text{l s}^{-1}$ through the chip. This flow rate was chosen to mimic the filling of the cell counting chambers by capillary action. In addition, we compared the release kinetics in the standard medium (PBS) with those in blood plasma and milliQ water. Blood samples were collected from healthy individuals at the University of Twente, all of whom had provided written informed consent in advance. All experiments were performed in compliance with the relevant laws and institutional guidelines. Fluorescence images of the cast layers were taken with 1 s exposure time at 5 s intervals. The excitation light was switched off between exposures to minimize bleaching. The fluorescence intensity of the layer was used to quantify the remaining IgG in the layer during the release process. Regions of interest (ROIs) of the layer with a specific thickness were selected. The thickness was calculated from the fluorescence intensity of the ROI and verified by white light interferometry in several cases. The fluorescence intensity of each of these ROIs was analyzed for all successive images to study the release kinetics. The statistical analysis of different release kinetics is described in the SI section “Statistical analysis of different release kinetics”.

3.2.8 Measurement of antibody distribution and intensity of stained cells in counting chambers

Gelatin/IgG layers with two different thicknesses, $0.25 \mu\text{m}$ and $0.5 \mu\text{m}$, were cast in cell counting chambers. A fluorescence image of each cell counting chamber (in the dry state) was recorded before $5 \mu\text{l}$ of blood was added to the filling port of the chamber and left to fill the chamber by capillary flow. Immediately after inflow a fluorescence image of the filled chamber was taken. The fluorescence intensity of a chamber without gelatin/IgG layer (autofluorescence of the chamber materials) was subtracted from the fluorescence images. Therefore, the represented intensities of the filled and unfilled chambers corresponded solely to the contribution of the fluorophore-labeled antibodies. For the pair of images before and after inflow, corresponding ROIs along the inflow direction were determined and the averaged intensity profiles were calculated across the length of the counting chamber. Then, the

intensity ratio of the filled chamber against the unfilled chamber at every position along the inflow direction was calculated. The normalized intensity profile is assumed to represent the ratio between antibody concentration after and before blood inflow. We also verified that the fluorescence intensity of IgG does not change significantly between the wet and the dry state and that the absorption of (red) excitation and emission light by blood can be neglected. After 15 min incubation, another image was taken and image analysis software (ImageJ²¹) was used to identify ROIs (a few pixels), which were assigned to target cells, and quantify the integrated intensity within each ROI, i.e. of each identified cell (after background subtraction).

3.3 Results and discussion

3.3.1 Diffusion-controlled release mechanism and the influence of layer thickness on the release

Gelatin/IgG layers with thicknesses between 0.25 μm and 1.5 μm were prepared under “standard conditions” (see above), cast on flow chambers. The fluorescence intensities of these gelatin layers, corresponding to the amount of IgG in the layer, were monitored during the release process. Fig. 3.4a shows the fractional release of IgG from gelatin layers. The percentage (M_t/M_{total}) of released antibody as a function of time was calculated using the IgG, released from the layer at time t , (M_t) and the total (initial) IgG, embedded in the layer (M_{total}). Clearly, the release of IgG from gelatin layers is delayed when compared to the control without gelatin, and the thicker the gelatin layer the slower the release. The data plotted as a function of the square root of time in Fig. 3.4a demonstrates that

$$\frac{M_t}{M_{\text{total}}} = \left(\frac{t}{\tau}\right)^{1/2} \quad (1)$$

during the initial phase of the release process²², which allows us to extract release times from the shown fits. The $t^{1/2}$ dependence is characteristic of Fickian diffusion, suggesting the initial release is diffusion-controlled.²³ The release time τ from a thin slab of material in a diffusion-controlled system can be expressed as²⁴:

$$\tau = \frac{\pi L^2}{4D} \quad (2)$$

where D is the diffusivity of IgG in the gelatin layer, and L is the layer thickness. The measured release kinetics demonstrates that D/L^2 is constant during the initial phase of the release process, which can be interpreted as a constant diffusivity following a much faster swelling process. The resulting diffusivities assuming instantaneous swelling to the equilibrium mass swelling ratio of ~ 13.5 (Fig. 3.5), determined from the water uptake of bulk gelatin, corresponding to a height ratio of 17.2, are given in Table 3.1. As the diffusivity is independent of the thickness of the dry layer, the measured release time is proportional to the square of the dry layer thickness (Fig. 3.4b), which allows us to tune release kinetics by choosing an appropriate layer thickness. The diffusivity of IgG in a swollen gelatin layer is two orders of magnitude lower than that in PBS^{25,26}, again, confirming that the release is delayed by embedding IgG in gelatin layers. Alternatively, instead of an instantaneous swelling followed by antibody release with a constant diffusivity, swelling and diffusion might also take place simultaneously with $D \propto L^2$, as expected (approximately) for a “jump diffusion”²⁷ mechanism. This case may require an alternative definition of the diffusivity, but would not affect the rest of the discussion.

Fig. 3.4: a) Representative release curves (symbols) of IgG from layers of varying thicknesses and their corresponding fits to $M_t/M_{\text{total}} = (t/\tau)^{1/2}$ (lines). The asterisk indicates “standard conditions” and the hash indicates the control without (additional) gelatin. b) Release time (τ) obtained from three independent release experiments as a function of the square of the thickness of the dry layer. Data points represent mean $\pm$ standard deviation ($n = 3$).

Table 3.1: Release time (τ) and diffusivity ($D_{\text{swollen layer}}$) of IgG in swollen gelatin layers with varying thicknesses. All values represent mean $\pm$ standard deviation ($n = 3$). As the 1.5 μm layers swell to almost the complete height of the chamber, the release from these layers was measured in both ~ 27 and ~ 52 μm high chambers and no significant differences were observed (Fig. 3.S2).

Thickness (μm) dry/swollen	τ (s)	$D_{\text{swollen layer}}$ ($\mu\text{m}^2 \text{s}^{-1}$)
0.25/4.3	23 $\pm$ 4	0.63 $\pm$ 0.10
0.50/8.6	88 $\pm$ 12	0.65 $\pm$ 0.08
0.75/12.9	176 $\pm$ 34	0.74 $\pm$ 0.11
1/17.2	392 $\pm$ 30	0.59 $\pm$ 0.03
1.5/25.8	647 $\pm$ 27	0.79 $\pm$ 0.04

3.3.2 Influence of layer preparation conditions on the release and possible mechanism

In diffusion-controlled release, the mobility of embedded molecules is dependent on the porosity of the hydrogel matrix.²⁸ It is described by the mesh size which is affected by the degree of (physical) crosslinking of hydrogels.²⁹ General physical crosslinking in hydrogels results from the random entanglement of disordered chains. In addition to these crosslinks, gelatin can take on a more ordered helical conformation involving three separate helical chains during the drying process³⁰, essentially increasing its degree of physical crosslinking. The formation of these triple helices and, thus, the degree of physical crosslinking of the gelatin layer can be influenced by the preparation conditions of gelatin layers. It has been reported that gelatin layers dried at 20°C or lower (“cold films”) contain collagen-like triple helices, while layers dried above 35°C (“hot films”) are solely comprised of random coils.^{31, 32} In the case of cold films, where triple helix formation is possible, higher humidity (contributing more water content in gelatin to stabilize helices via hydrogen bonds³⁰) and longer drying times (allowing for the slow helix formation process³³) promote helix formation.

The mass swelling ratio (between swollen and dry state) of hydrogels is known to strongly depend on the average distance between crosslinks (given as average mass of monomers between crosslinks), i.e. the mesh size.^{29, 34} This makes the swelling ratio an excellent measure of the conformational state of gelatin with regard to its degree of crosslinking due to triple helix formation.³⁵ We therefore determined the mass

swelling ratio of gelatin layers prepared under different conditions, for which different degrees of crosslinking were to be expected.

Fig. 3.5 shows that layers dried at lower temperature and higher RH show a smaller equilibrium mass swelling ratio, indicating smaller pore size and a larger degree of crosslinking, in agreement with expectations as well as previous findings.³⁰ This change of conformation with changing temperature and humidity was further corroborated by Fourier Transform Infrared (FT-IR) spectroscopy (Fig. 3.S4 and Table 3.S1).

In order to study the effect of this gelatin-intrinsic type of crosslinking on reagent release, we examined the release of antibody from cast gelatin layers dried under different conditions. And indeed, as shown in Fig. 3.6, layers dried at higher temperature show faster release, probably due to a lower degree of physical crosslinking as a consequence of reduced formation of triple helices in layers dried at higher temperatures.

Fig. 3.5: Equilibrium mass swelling ratios of gelatin layers prepared under different temperatures and humidities. Data represents mean $\pm$ standard deviation ($n = 4$)

Fig. 3.6: Representative release curves (symbols) of IgG from 0.5 μm thick gelatin layers dried at different temperatures and fits of the initial release according to Eq. (1) (lines). The asterisk indicates “standard conditions”. Release times (τ) are values for the specific release experiments shown here.

Likewise in Fig. 3.7, layers dried in a humidity chamber ($\sim 85\%$ RH), indeed show a slower release than layers dried under ambient atmosphere ($\sim 40\%$ RH), confirming that the formation of triple helices is very likely to play a role in the fine-tuning of release characteristics of gelatin layers. In addition, it is noticeable in Fig. 3.7, that the release from layers dried *in vacuo* is much faster than the release from layers prepared under ambient pressure. This may be ascribed to a lower degree of physical crosslinking of gelatin due to faster drying and lower water content compared to the ambient atmosphere. The formation of triple helices in gelatin is believed to be a process taking place on a timescale of many hours.³³ Therefore it is not surprising that drying *in vacuo*, taking about 5 min, is too short to allow for considerable formation of triple helices. In contrast, ambient ($\sim 40\%$ RH) and humid ($\sim 85\%$ RH) atmosphere maintain sufficient moisture content in the gelatin and extend the drying period to approximate 30 min and 2 days, respectively, thus facilitating extensive formation of triple helices.

Fig. 3.7: Representative release curves (symbols) of IgG from 0.5 μm thick layers dried under reduced pressure (0.2 bar, black circles), ambient atmosphere (blue triangles) and 85% RH (green squares) and fits of the initial release according to Eq. (1) (lines). The asterisk indicates “standard conditions”. Release times (τ) are values for the specific release experiments shown here.

Table 3.2 summarizes the release times in gelatin layers prepared under different conditions.

In conclusion, low temperature, high humidity and long drying period, result in slower release and are probably due to the formation of a larger amount of triple helices, increasing the degree of physical crosslinking and thus decreasing the mesh size.

Table 3.2: Release times (τ) of IgG in swollen gelatin layers with initial thickness of 0.5 μm dried under different conditions. All values represent mean $\pm$ standard deviation ($n = 3$).

T(°C)	atmosphere	τ (s)
20	85% RH	194 $\pm$ 14
20	40% RH	88 $\pm$ 12
35	40% RH	46 $\pm$ 4
40	40% RH	35 $\pm$ 1
20	<i>in vacuo</i>	N.A.

3.3.3 Influence of reagent size on the release

In addition to the structure of the release layer, the size of released reagent also has an impact on diffusion-controlled release.³⁶⁻⁴⁰ Four types of antibody and antibody

fragments with different molecular weights (MWs) were chosen to study the influence of the reagent size on the release. The radii of the antibodies were calculated using

$$r[\text{nm}] = 0.066 \times (\text{MW}[\text{Da}])^{1/3} \quad (3)$$

which assumes the antibody to be spherical, having a density of 1.38 g cm^{-3} .⁴¹ Gelatin layers were prepared under “standard conditions”. Fig. 3.8a clearly shows that the larger the protein the slower the diffusion. The $t^{1/2}$ dependence in the initial phase of the release process points at a Fickian diffusion-controlled release behavior. Fig. 3.8b shows that there is an inverse correlation between diffusivity and molecular radius. The observed influence of molecular size on the diffusivity is in agreement with the findings obtained from other collagen^{42, 43} and gelatin⁴⁴ based release systems.

Fig. 3.8: a) Representative release curves (symbols) of antibodies with different MWs from $0.5 \mu\text{m}$ thick layers and fits of their initial release according to Eq. (1) (lines). The asterisk indicates “standard conditions”. Release times (τ) are values for the specific release experiments shown here. b) Diffusivity ($D_{\text{swollen gel}}$) of antibodies and antibody fragments. Data points represent mean $\pm$ standard deviation ($n = 3$).

3.3.4 Influence of electrostatic interactions on the release

Electrostatic interactions between gelatin and any embedded antibody influence the release kinetics of antibodies from the gelatin matrix and can be influenced by the physicochemical properties of the release medium.⁴⁵ Therefore, blood plasma, PBS and milliQ water were compared as release media to study the influence of the medium on the release of IgG from gelatin layers. As shown in Fig. 3.9, the release times of IgG in PBS ($82 \pm 15 \text{ s}$, $n = 3$) and blood plasma ($82 \pm 10 \text{ s}$, $n = 3$) are similar to each other and much shorter than in milliQ water. Additionally, considerably more IgG seems to stay trapped in the layer in milliQ water compared to PBS and plasma. Since

both gelatin and IgG contain charged functional groups, the ions in PBS are presumed to screen surface charges, reducing the interaction between gelatin and antibodies, thereby favoring the detachment of antibody from the gelatin matrix. In milliQ water, such screening is much reduced, which should result in longer release times (312 ± 51 s, $n = 3$) and less antibody release, which is indeed the case. The finding that antibody release in blood plasma and PBS are comparable supports this explanation, as the ion concentration in blood plasma is similar to that in PBS. These results also prove that PBS is a suitable medium to replace blood plasma in release studies.

Fig. 3.9: Representative release curves (symbols) of IgG from $0.5 \mu\text{m}$ thick layers in blood plasma (red squares), PBS (blue triangles) and milliQ water (green squares) and fits of their initial release according to Eq. (1) (lines). The asterisk indicates “standard conditions”. Release times (τ) are values for the specific release experiments shown here.

3.3.5 Implication of antibody release kinetics for on-chip cell staining.

Finally, we proceeded to verify that the expected release kinetics has an immediate effect on the antibody distribution in the final application, a cell counting chamber after blood inflow, and consequently can help to improve the quality of cell staining. We compared a cell counting chamber containing a $0.25 \mu\text{m}$ thick gelatin layer with an expected fast release ($\tau = 20$ s) with one containing a $0.5 \mu\text{m}$ thick layer with an expected slow release ($\tau = 88$ s). The measured ratios between antibody

concentrations after and before blood inflow, plotted against the distance from the filling port, are shown in Fig. 3.10a. Clearly, the 0.5 μm thick layer shows a much flatter intensity profile than the 0.25 μm layer, i.e. the 0.5 μm thick layer prevents wash-off during blood inflow, which takes about 5-10 s, much better than the 0.25 μm thick layer. In the case of fast release from a 0.25 μm thick layer, the inflowing blood washes off about 70% of the antibody close to the filling port and transports it towards the venting port, where the antibody concentration roughly doubles. In contrast, the antibody in a chamber with a 0.5 μm thick layer changes by less than 20% throughout the whole length of the chamber. To illustrate that the improvement in antibody distribution directly translates into improved cell staining, we determine the fluorescence intensities of T-lymphocytes (CD3^+) after 15 min incubation in the “filling region” (0-1 mm from the filling port) and the “venting region” (0-1 mm from the venting port) and translate this intensity to the number of IgGs bound per cell by means of the calibration which is described in Chapter 2. Histograms of the number of antibody molecules bound per cell in these regions are shown in Fig. 3.10b. The increased antibody concentration close to the venting port in the chamber with the 0.25 μm layer does not result in significantly increased fluorescence intensity per cell (saturation) and it only reduces the signal-to-background ratio, as the simple design of the cell counting chambers does not allow for a washing step. In contrast, the reduced concentration close to the filling port results in significantly reduced cell intensities. As a consequence of the relatively homogeneously distributed antibody in the chamber with the 0.5 μm thick layer, cell intensities are homogeneous throughout the full length of the chamber. The obtained saturation intensity matches well with the reported CD3 antibody binding capacities on T-cells between 60k and 70k⁴⁶. Using the calibration of fluorescence intensity against antibody concentration (see Chapter 2), we can quantitatively monitor the progress of antibody binding in real time. Quantitative information on the saturation is of great use to assess and later fine-tune assay parameters, such as antibody concentration, incubation time or minimum needed and maximum attainable cell intensities.

Fig. 3.10: a) IgG distribution in cell counting chambers with a 0.25 μm (red) and a 0.5 μm thick (blue) layer after blood inflow. Dashed squares and solid rectangles denote the “filling region” and “venting region”, respectively. b) Histograms of the amount of bound antibodies per cell at the “filling region” (left panel) and “venting region” (right panel) in the chambers with 0.25 μm (red) and 0.5 μm (blue) thick gelatin layers after 15 min incubation. The insets are zoom-in fluorescence images of stained cells from a representative area in the “filling and venting regions”. The white dots are identified cells. Intensity scale: 0 (black) to 2000 camera units per second/CPS (white). Scale bars 50 μm .

To briefly summarize, we have shown the close correlation between the antibody release time, the resulting antibody distribution across our cell counting chambers and thus, the homogeneity of cell staining. By choosing preparation conditions

judiciously, based on this study, optimal cell staining for our cell counting application can be achieved.

3.4 Conclusion

We have studied the release kinetics of antibodies embedded in gelatin layers with (sub-) μm -scale thicknesses on second to minute timescales. The gelatin layers were monitored by real-time and *in situ* fluorescence imaging during the release in a flow chamber. Under most conditions, the initial phase of antibody release from cast layers is proportional to $t^{1/2}$, indicating diffusion-controlled release. The quadratic dependence of the release time on the thickness of the dry layer confirms this mechanism. The release is strongly influenced by the size of the antibody or antibody fragment in the gelatin layer and by the ion content of the medium. By varying the temperature, humidity and drying time during the preparation of gelatin matrices for reagent release, we can influence the material properties of the layer and thereby control reagent release times. In the final application, a cell counting chamber, the release time is shown to determine the antibody distribution throughout the chamber, which in turn governs the spatial distribution of cell staining intensities. This proves that the knowledge of the release kinetics in flow chambers, can be translated into the performance of sample-reagent mixing in cell counting chambers. The insights gained here, on how to tune release times of antibodies from gelatin layers enable us to tailor on-chip sample preparation in cell counting chambers. Moreover, the concept of this study can be extended to investigate on-chip release of a variety of reagents (e.g. lysis agents, fluorescent dyes, enzymes and drugs) from a hydrogel matrix. The understanding of the release kinetics of these reagents can be translated to tune the sample-reagent mixing in a multitude of microfluidic devices and optimize on-chip sample preparation for many biological assays.

3.5 Supporting information

3.5.1 Reproducibility of the gelatin layers prepared using “standard conditions” and chamber heights

11 gelatin layers prepared in independent experiments by using “standard conditions” were compared. A ROI was defined in the central region of each layer. The average thicknesses and the fluorescence intensities of each ROI are shown in Fig. 3.S1. A good reproducibility of layer preparation is achieved, evidenced by the coefficient of variation of thickness and fluorescence intensity of 4.1% and 2.7%, respectively. The height of the chamber was measured by laser interferometry. The heights of sample chambers assembled by single and double layers of laminating adhesive were $26.6 \pm 0.4 \mu\text{m}$ ($n = 9$) and $52.3 \pm 0.5 \mu\text{m}$ ($n = 9$), respectively.

Fig. 3.S1: Thickness and fluorescence intensity in the central region of layers prepared using “standard conditions”. Each black open square is the average result of a ROI in a specific layer. The red square with error bars represent mean $\pm$ standard deviations ($n = 11$).

3.5.2 Exclusion of perfusion in the release process

0.25 μm and 1.5 μm thick layers were prepared and release experiments on these layers using different flow rates were performed. As shown in Fig. 3.S2, the minor influence of the flow rate on the release kinetics of 0.25 μm and 1.5 μm thick layers in ~ 27 μm chambers suggests that perfusion plays a minor role in the release. Since 1.5 μm thick layers can swell to about 26 μm which is very close the height of the chamber with a single layer of laminating adhesive, the release kinetics of 1.5 μm thick layers in the chambers with different heights (~ 27 μm and ~ 52 μm) were compared. No significant differences of the release kinetics were observed. The fact, that the chamber height has hardly any influence on the release kinetics, indicates that the release takes place directly at the interface between the layer surface and eluting medium, even if there is only a very small volume of eluting medium left above the hydrogel layer. Since we were testing the influence of flow rate individually on the layer with the same thickness in the same chamber (i.e. the same free volume in the chamber for fluid traffic), it is still fair to quantify the flow rate using volume scale (instead of velocity scale) to draw the conclusion. Meanwhile, we do not doubt that a more striking evidence will be shown (i.e. comparison between Fig. 3S2a and 3S2b) using velocity scale to quantify the flow rate.

Fig. 3.S2: Influence of flow rates on release kinetics. a) 0.25 μm thick layer, chamber height: $\sim 27 \mu\text{m}$. b) 1.5 μm thick layer, chamber height: $\sim 27 \mu\text{m}$. c) 1.5 μm thick layer, chamber height: $\sim 52 \mu\text{m}$.

3.5.3 Statistical analysis of different release kinetics

To determine the significance of release time and diffusivity discussed in the manuscript, all the data were analyzed using ANOVA with subsequent Tukey test using SPSS 23. In Fig. 3.S3a, decreased temperature and elevated humidity significantly increase the release time, while a significant reduction of release time is observed when the eluting medium is changed from MQ to PBS or plasma. Fig. 3.S3b shows a significant inverse correlation between D and r . Although the results of normality test and homogeneity of variances test in SPSS show the samples within each condition group (temperature, RH, size of reagent, media) are normally distributed with equal variance, we remain cautious about the eligibility of the test and the significance claim, due to the limited sample size.

Fig. 3.S3: a) Left: Release times (τ) of IgG from gelatin layers prepared under different conditions in PBS, Right: Release times (τ) of IgG from gelatin layers prepared under “standard conditions” in different media. Data points represent mean $\pm$ standard deviation ($n = 3$). The symbol (*) indicates a significant difference ($p < 0.05$) in release time (τ) between the compared conditions. b) Diffusivity ($D_{\text{swollen layer}}$) of antibodies and antibody fragments in gelatin layers prepared under “standard conditions”. Data points represent mean $\pm$ standard deviation ($n = 3$). The symbol (*) indicates a significant difference ($p < 0.05$) in diffusivity ($D_{\text{swollen layer}}$) between compared reagents.

3.5.4 FT-IR spectroscopy

A 0.45% w/v gelatin solution was prepared. 50 μL of the solution was cast in areas of 10 mm $\times$ 10 mm on gold substrates. Two drying conditions, $\sim 85\%$ RH, 4°C and $\sim 40\%$ RH, 45°C , were applied to obtain “cold” and “hot” gelatin layers, respectively. The infrared absorption spectra of gelatin layers were acquired using a Nicolet 6700 ATR-FT-IR spectrometer (ThermoScientific).

Fig. 3.S4: Representative FT-IR spectra of gelatin layers prepared under different conditions.

Differences in absorption at a characteristic band, 1240 cm^{-1} assigned to triple helix formation⁴⁷, were observed when comparing “hot” and “cold” films. The 1240 cm^{-1} peak height was normalized with respect to the 1450 cm^{-1} band, associated with the protein backbone and as such not sensitive to conformational changes. It can be clearly seen in Table 3.S1 that for samples prepared at high temperature (45°C) the $1240\text{ cm}^{-1}/1450\text{ cm}^{-1}$ ratios are much lower than for samples prepared at lower temperature (4°C) corroborating our assumption that we can fine tune materials properties by changing the conditions under which layers are prepared.

Table 3.S1: FT-IR absorption ratios of A_{1240}/A_{1450} (mean $\pm$ standard deviation, $n = 3$) for gelatin layers dried under different conditions.

drying conditions	A_{1240}/A_{1450}
85% RH 4°C	0.58 ± 0.03
40% RH 45°C	1.58 ± 0.16

References

1. W. Weaver, H. Kittur, M. Dhar and D. Di Carlo, *Lab Chip*, 2014, 14, 1962-1965.
2. C. D. Chin, V. Linder and S. K. Sia, *Lab Chip*, 2012, 12, 2118-2134.
3. V. Gubala, L. F. Harris, A. J. Ricco, M. X. Tan and D. E. Williams, *Anal. Chem.*, 2012, 84, 487-515.
4. L. Gervais, N. de Rooij and E. Delamarche, *Adv. Mater.*, 2011, 23, H151-H176.
5. M. Hitzbleck and E. Delamarche, *Chem. Soc. Rev.*, 2013, 42, 8494-8516.
6. B. Weigl, G. Domingo, P. LaBarre and J. Gerlach, *Lab Chip*, 2008, 8, 1999-2014.
7. T. van Oordt, Y. Barb, J. Smetana, R. Zengerle and F. von Stetten, *Lab Chip*, 2013, 13, 2888-2892.
8. D. Czurratis, Y. Beyl, A. Grimm, T. Brettschneider, S. Zinober, F. Larmer and R. Zengerle, *Lab Chip*, 2015, 15, 2887-2895.
9. R. Boden, M. Lehto, J. Margell, K. Hjort and J. A. Schweitz, *J. Micromech. Microeng.*, 2008, 18, 075036.
10. J. Hoffmann, D. Mark, S. Lutz, R. Zengerle and F. von Stetten, *Lab Chip*, 2010, 10, 1480-1484.
11. D. Y. Stevens, C. R. Petri, J. L. Osborn, P. Spicar-Mihalic, K. G. McKenzie and P. Yager, *Lab Chip*, 2008, 8, 2038-2045.
12. E. Fu, B. Lutz, P. Kauffman and P. Yager, *Lab Chip*, 2010, 10, 918-920.
13. M. Hitzbleck, L. Gervais and E. Delamarche, *Lab Chip*, 2011, 11, 2680-2685.
14. S. Q. Jin, M. H. Dai, B. C. Ye and S. R. Nugen, *Microsyst. Technol.*, 2013, 19, 2011-2015.
15. L. Gervais and E. Delamarche, *Lab Chip*, 2009, 9, 3330-3337.
16. J. Kim, D. Byun, M. G. Mauk and H. H. Bau, *Lab Chip*, 2009, 9, 606-612.
17. M. Beck, S. Brockhuis, N. van der Velde, C. Breukers, J. Greve and L. W. M. M. Terstappen, *Lab Chip*, 2012, 12, 167-173.
18. M. Mortato, L. Blasi, G. Barbarella, S. Argentiore and G. Gigli, *Biomicrofluidics*, 2012, 6, 044107.
19. A. N. Zelikin, *ACS Nano*, 2010, 4, 2494-2509.
20. GMIA, Gelatin Handbook, *Gelatin Manufacturers Institute of America*, New York, 2012.
21. C. A. Schneider, W. S. Rasband and K. W. Eliceiri, *Nat. Methods*, 2012, 9, 671-675.
22. T. Higuchi, *J. Pharm. Sci.*, 1961, 50, 874-875.
23. H. L. Frisch, *Polym. Eng. Sci.*, 1980, 20, 2-13.
24. J. Siepmann, R. Siegel, A. and M. Rathbone, J., *Fundamentals and Applications of Controlled Release Drug Delivery*, Springer, New York, 2012.
25. M. L. Radomsky, K. J. Whaley, R. A. Cone and W. M. Saltzman, *Biomaterials*, 1990, 11, 619-624.
26. E. A. Schnell, L. Eikenes, I. Tufto, A. Erikson, A. Juthajan, M. Lindgren and C. de Lange Davies, *J. Biomed. Opt.*, 2008, 13, 064037.
27. N. A. Peppas and C. T. Reinhart, *J. Membr. Sci.*, 1983, 15, 275-287.
28. Y. Wu, S. Joseph and N. R. Aluru, *J. Phys. Chem. B*, 2009, 113, 3512-3520.
29. C. C. Lin and A. T. Metters, *Adv. Drug Delivery Rev.*, 2006, 58, 1379-1408.
30. P. V. Kozlov and G. I. Burdygina, *Polymer*, 1983, 24, 651-666.
31. E. Bradbury and C. Martin, *Proc. R. Soc. Lond. A Math. Phys. Sci.*, 1952, 214, 183-192.
32. M. Djabourov and P. Papon, *Polymer*, 1983, 24, 537-542.
33. S. B. Rossmurphy, *Polymer*, 1992, 33, 2622-2627.

34. M. N. Mason, A. T. Metters, C. N. Bowman and K. S. Anseth, *Macromolecules*, 2001, 34, 4630-4635.
35. A. Bigi, S. Panzavolta and K. Rubini, *Biomaterials*, 2004, 25, 5675-5680.
36. M. Henke, F. Brandl, A. M. Goepferich and J. K. Tessmar, *Eur. J. Pharm. Biopharm.*, 2010, 74, 184-192.
37. N. K. Reitan, A. Juthajan, T. Lindmo and C. de Lange Davies, *J. Biomed. Opt.*, 2008, 13, 054040.
38. F. Brandl, F. Kastner, R. M. Gschwind, T. Blunk, J. Tessmar and A. Goepferich, *J. Control. Release*, 2010, 142, 221-228.
39. N. Fatin-Rouge, K. Starchev and J. Buffle, *Biophys. J.*, 2004, 86, 2710-2719.
40. A. A. Dinerman, J. Cappello, H. Ghandehari and S. W. Hoag, *J. Control. Release*, 2002, 82, 277-287.
41. H. P. Erickson, *Biol. Proced. Online*, 2009, 11, 32-51.
42. T. Kihara, J. Ito and J. Miyake, *PLoS One*, 2013, 8, e82382.
43. D. L. Gilbert, T. Okano, T. Miyata and S. W. Kim, *Int. J. Pharm.*, 1988, 47, 79-88.
44. Y. E. Fang, Q. Cheng and X. B. Lu, *J. Appl. Polym. Sci.*, 1998, 68, 1751-1758.
45. Y. Tabata and Y. Ikada, *Adv. Drug Delivery Rev.*, 1998, 31, 287-301.
46. L. Ginaldi, M. De Martinis, A. D'Ostilio, L. Marini, F. Loreto, M. Modesti and D. Quaglino, *Am. J. Hematol.*, 2001, 67, 63-72.
47. P. L. Gordon, C. Huang, R. C. Lord and I. V. Yannas, *Macromolecules*, 1974, 7, 954-956.

Chapter 4

Temperature switch cytometry – releasing antibody on demand from inkjet-printed gelatin for on-chip immunostaining

Xichen Zhang, Dorothee Wasserberg, Christian Breukers, Leon W. M. M. Terstappen, Markus Beck

This chapter has been published as:
ACS Appl. Mater. Interfaces, 2016, 8, 27539–27545

Abstract

Complete integration of all sample preparation steps in a microfluidic device greatly benefits point-of-care diagnostics. In the most simplistic approach, reagents are integrated in a microfluidic chip and are dissolved upon filling with a sample fluid by capillary force. This will generally result in at least partial reagent wash-off during sample inflow. However, many applications, such as immunostaining based image cytometry, strongly rely on a homogeneous reagent distribution across the chip. The concept of initially preventing release (during inflow), followed by a triggered instantaneous and complete release on demand (after filling is completed) represents an elegant and simple solution to this problem. Here, we realize this controlled release by embedding antibodies in a gelatin layer integrated in a microfluidic chamber. The gelatin/antibody layer is deposited by inkjet printing. Maturation of this layer during the course of several weeks, due to the ongoing physical crosslinking of gelatin, slows down the antibody release, thereby reducing antibody wash-off during inflow, and consequently helping to meet the requirement for a homogeneous antibody distribution in the filled chamber. After inflow, complete antibody release is obtained by heating the gelatin layer above its sol-gel transition temperature, which causes the rapid dissolution of the entire gelatin/antibody layer at moderate temperatures. We demonstrate uniform and complete on-chip immunostaining of CD4 positive (CD4⁺) T-lymphocytes in whole blood samples, which is critical for accurate cell counts. The sample preparation is realized entirely on-chip, by applying temperature switched antibody release from matured gelatin/antibody layers.

4.1 Introduction

Microfluidics technology enables the miniaturization of in-vitro diagnostics. A microfluidic device with fully integrated on-chip sample preparation would facilitate diagnostic applications of point-of-care devices tremendously.¹⁻⁴ To achieve this goal, reagents have to be integrated in the chip; subsequent sample delivery and on-chip processing have to be simple with minimal required manual intervention and minimum reliance on peripheral equipment.⁵⁻⁹ Hydrogel matrices have long been proven to be promising carriers of embedded pharmaceutical reagents for their release in a controlled manner in diverse drug delivery applications.¹⁰ Recently, more and more hydrogel materials are implemented in microfluidic chips as matrices to achieve controlled reagent release upon contact with inflowing sample fluid.^{11, 12} For a simple application in a chip with a stopped flow configuration, switchable release from a tunable hydrogel matrix is essential in preventing release during sample inflow and starting release on demand after inflow has stopped. This will effectively prevent reagent wash-off and ensure homogeneous reagent distribution across the complete chip.

We previously demonstrated on-chip sample preparation for the enumeration of CD4⁺ T-lymphocytes (CD4 count) in point-of-care settings.¹¹ In that application, sample preparation is realized in a straightforward manner: Whole blood flows into a gelatin coated counting chamber by capillary action. Fluorophore-labeled antibodies (in this assay, APC- α CD3 is used to target all T-lymphocytes, PerCP- α CD4 is used to target the CD4⁺ subpopulation of the T-lymphocytes) are released from gelatin, only after a certain level of swelling has been reached upon blood inflow, resulting in the immunostaining of the cells of interest in the sample. Fluorescence imaging and automated image analysis yields the cell count. To ensure sufficient sample volume ($>1 \mu\text{L}$) per image in a chamber with a defined height ($\sim 27 \mu\text{m}$), a wide field of view ($7.8 \text{ mm} \times 5.2 \text{ mm}$) imaging setup has been designed. Due to its low optical resolution ($\sim 10 \mu\text{m}$), stained cells have to be identified based on their fluorescence intensities only, which makes uniform intensities of stained cells as well as homogeneous background intensities essential.

In our previous CD4 counting application, all antibody release layers were

fabricated by manually casting a gelatin/antibody solution. Though the material cost is low, the production was labor-intensive and the reproducibility depended strongly on skills and experience of the operator. To overcome these limitations, we employed inkjet printing to deposit gelatin/antibody layers in cell counting chambers. Inkjet printing is a powerful tool to deposit polymers in a cost-effective and reproducible manner.¹³⁻¹⁵ It has been widely used in functional material fabrication for drug encapsulation¹⁶, biomolecule preservation¹⁷ and biosensing¹⁸⁻²⁰. To optimize the on-chip cell staining, we studied the release of fluorophore-labeled antibody from differently prepared gelatin layers. It has been shown that gelatin experiences gradually increasing physical crosslinking during the course of maturation.^{21, 22} Consistently, during the course of maturation, we observed a deceleration of the antibody release and increasing fraction of antibody trapped in the gelatin matrix, which we interpret as an increase in physical crosslinking. To determine the optimal maturation conditions for the desired antibody release kinetics, we carried out release experiments, in which a medium was continuously passed through a flow chamber coated with a gelatin/antibody layer. The release of fluorophore-labeled antibody was determined from the decrease in fluorescence intensity of the gelatin layer, which was monitored over time. From the release experiments, we found an optimal condition (2-step maturation) to efficiently generate matured gelatin that sufficiently delays antibody release. In addition, we demonstrated that heating gelatin layers above their sol-gel transition temperature initiates rapid and complete antibody release on demand. By translating this into the final application, optimized on-chip sample preparation in cell counting chambers fabricated by inkjet printing was achieved. Optimal maturation of gelatin layers prevents antibody wash-off during blood inflow for homogenous cell staining, while temperature switched release after blood inflow allows for optimized cell staining, as all antibody becomes available immediately.

4.2 Experiments

4.2.1 Flow chamber and cell counting chamber fabrication

As shown in Fig. 4.1, both flow chambers and cell counting chambers were assembled using poly(methyl methacrylate) (PMMA) substrates (76 mm × 26 mm × 1 mm) for the

deposition of gelatin/antibody layers, laminating adhesive (nominal thickness $25.4\ \mu\text{m}$, 3M) as spacer material and capping glass slides (Menzel, $76\ \text{mm} \times 26\ \text{mm} \times 1\ \text{mm}$). Flow chambers ($61\ \text{mm} \times 4.8\ \text{mm}$) and counting chambers ($15\ \text{mm} \times 9\ \text{mm}$) were created as cut-outs in the laminating adhesive and then attached to the PMMA substrates. Following the deposition of gelatin/antibody by inkjet printing (see next section) on the substrates, capping slides with holes to connect tubing were attached to the coated substrates, completing the flow chambers. For counting chambers, the substrates were covered by capping slides ($76\ \text{mm} \times 11\ \text{mm} \times 1\ \text{mm}$) to create openings to allow for filling in sample solution in and venting air. Chamber heights determined by laser interferometry were found to be $26.6 \pm 0.4\ \mu\text{m}$ ($n = 9$). A more detailed description of the fabrication procedure can be found elsewhere.²³

Fig. 4.1: Schematic representations of a flow chamber (left panel) and a cell counting chamber (right panel). a), c) Perspective view and b), d) Side view. The color gradient in c) indicates the distribution of antibody after blood inflow: left chamber, for a case of strong wash-off; right chamber: the homogeneous distribution for a case using matured gelatin layers.

4.2.2 Inkjet printing and layer maturation

To prepare the ink, gelatin powder (type A, Bloom 295 g, Sigma-Aldrich) was dissolved in milliQ water at 40°C for 1 h while stirring. A 0.3% w/v gelatin solution containing $0.6\ \mu\text{g mL}^{-1}$ allophycocyanin labeled antiCD3 IgG (APC- αCD3 , clone SK7, $\sim 260\ \text{kDa}$, BD) and $0.2\ \mu\text{g mL}^{-1}$ peridinin-chlorophyll labeled antiCD4 IgG (PerCP- αCD4 , clone MEM241, $\sim 308\ \text{kDa}$, Exbio) was filtered gravitationally using a membrane filter (CellTrics®, mesh size $20\ \mu\text{m}$, Partec) before use. Inkjet printing was performed using

an industrial inkjet printer (LP50, PixDro, Meyer Burger B.V.) with a Konica Minolta 512MHX printhead. Droplets of ~ 20 pL gelatin/antibody solution were dispensed in 9 swaths at $(360 \text{ dpi})^2$ resolution each and a delay of 84 s between them, resulting in a final resolution of $(1080 \text{ dpi})^2$. These parameters, resulting in an average layer thickness of ~ 80 nm after drying (dispensed volume per area: $0.036 \mu\text{L mm}^{-2}$ gelatin content: $0.003 \text{ mg } \mu\text{L}^{-2}$, density of gelatin: 1.3 g cm^{-3}), were chosen to minimize puddle formation while maintaining sufficient throughput to keep nozzles jetting reliably.

In order to determine optimal maturation conditions, freshly printed chambers were kept at 4°C either in containers with $\sim 85\%$ relative humidity (RH) maintained above a saturated potassium chloride solution, or above silica gel resulting in $\sim 10\%$ RH for maturation. Later, a 2-step procedure (initial maturation for 2 days under 85% RH and follow-up maturation under 10% RH up to 6 weeks) scheme was tested.

4.2.3 Layer characterization

The topography of gelatin/antibody layers was determined using a white light interferometer (smartWLI-microscope, GBS). The distribution of fluorophore-labeled antibody within the printed gelatin layers was characterized using fluorescence images of the layers, which were taken by our custom-built fluorescence imaging system described previously.¹¹ Fig. 4.2a and 4.2b show the topography and fluorescence readout (APC- αCD3) from the same region of a representative printed gelatin/antibody layer, respectively. The distributions of layer thickness and fluorescence intensity resemble each other very closely, indicating a homogeneous mixture of gelatin and antibody across the printed layer.

Fig. 4.2: a) Contour plot. b) Fluorescence (APC-αCD3 readout) image of the same region of a dry, printed gelatin/antibody layer.

4.2.4 Measurement of release kinetics in flow chambers

Phosphate buffered saline (PBS) was used as medium flowing through our flow chambers at a rate of $1 \mu\text{L s}^{-1}$. This flow rate was chosen to mimic the filling of the counting chambers by capillary action. Fluorescence images of the printed layers were continuously taken at a 2 s exposure time and at 6 s intervals. The excitation light was switched off between exposures to minimize bleaching. The fluorescence intensity of the layer was used to quantify the amount of antibody remaining in the layer during the release process. As the differences between the release kinetics of the two antibodies are negligible (Fig. 4.S4 shows a comparison between the release kinetics of APC-αCD3 and PerCP-αCD4 for two conditions.), the release experiments were mainly performed on APC-αCD3. For each release experiment, a region in the layer was selected at random, as region of interest (ROI). The intensities of each of these ROIs obtained from time-lapse images were analyzed to study the release kinetics. Release experiments were generally performed at room temperature (RT). Only exception was the temperature switched release experiments, for which the flow chambers were placed on a temperature controllable heating stage. The stage was

kept at RT during the first minute of release, to establish a reference, after which the temperature controller of the stage was set to 40°C to start heating. The stage was kept at 40°C for 3 minutes after this temperature had been reached, then, the heating was switched off. In order to quantify the effect of release kinetics on cell staining, we determined two characteristic release fractions after 10 s ($M_{10\text{ s}}/M_{\text{total}}$) and 10 min ($M_{10\text{ min}}/M_{\text{total}}$) from the time of inflow. To prevent wash-off of antibody during sample inflow in the counting chamber, $M_{10\text{ s}}/M_{\text{total}}$ should be minimized, whereas $M_{10\text{ min}}/M_{\text{total}}$ should be maximized to ensure optimal cell staining within the 40 min incubation time.

4.2.5 Measurement of antibody distribution and intensity of stained cells in cell counting chambers

Cell counting chambers containing printed layers subjected to different maturation conditions were prepared. Blood samples were collected from anonymous and healthy individuals at the University of Twente, all of whom had provided written informed consent in advance. All experiments were performed in compliance with the relevant laws and institutional guidelines. The influence of blood inflow on antibody distribution was tested in the following manner: A fluorescence image of each chamber (in the dry state) was taken before 5 μL of the blood sample was added to the filling port of the chambers and left to fill the chambers by capillary flow. Immediately after inflow, a fluorescence image of each filled chamber was taken. The fluorescence intensity (APC- αCD3 readout) of a chamber without gelatin/antibody layer (autofluorescence of the materials) was subtracted from all fluorescence images. As described in Chapter 3, normalized intensity profiles were obtained from the ratios of fluorescence intensities of the filled versus the unfilled chamber at each corresponding position along the inflow direction in the autofluorescence-corrected images. These normalized profiles represent the ratio between antibody concentrations after versus before blood inflow.

Generally, on-chip immunostaining was conducted at RT, however, for the temperature switched case, filled chambers were placed on a 40°C heating stage for 3 min. Fluorescence (APC- αCD3 readout) images of both heated and unheated chambers were recorded. After an additional 37 min of incubation, another set of fluorescence

images with both APC- α CD3 and PerCP- α CD4 readout was taken and automated image analysis using ImageJ²⁴ was performed to identify ROIs (2-9 pixels in diameter) with the fluorescence intensities exceeding the respective background level significantly. Each ROI (i.e. each identified cell) was quantified with regard to its integrated intensity of both fluorophores after background subtraction. ROIs with above-threshold fluorescence intensities for both fluorophores were assigned to be CD4⁺ T-lymphocytes.

4.3 Results and discussion

4.3.1 Delayed antibody release induced by layer maturation

Antibody release from gelatin layers was discovered to be strongly dependent on the conformation of the gelatin matrix.²³ Freshly printed layers are comprised of small drops of gelatin, which are separately dispensed, and therefore dry so rapidly that mainly random entanglement between gelatin chains occurs and additional physical crosslinking via triple helix formation is suppressed.^{23,25} Such rapidly dried gelatin will undergo continuous physical crosslinking via triple helix formation in a long-term structural evolution towards full maturation.^{21, 22} The increased degree of physical crosslinking in gelatin suggests a reduced mesh size in the gelatin matrix, thus causing an increased delay in antibody release. Obviously, optimal maturation conditions are essential to obtain the desired antibody release kinetics. Low temperatures (<20°C) and high humidity lead to a rapid increase of the degree of physical crosslinking during maturation.^{21, 22, 26-28} We indeed find that high humidity (85% RH) is more efficient than dry conditions (10% RH) in delaying the release of antibodies (Fig. 4.S1a). This seems to confirm that humidity promotes the physical crosslinking of gelatin.

However, prolonged maturation under humid conditions results in a considerable reduction of the stability of the embedded fluorophores, which are more stable under dry conditions (Fig. 4.S1b). Therefore, we adopted a 2-step maturation procedure, i.e. initial maturation at 4°C under 85% RH for 2 days and follow-up maturation at 4°C under 10% RH up to 6 weeks. Step 1, initial maturation, ensures rapid physical crosslinking for a profound delay in release and step 2, follow-up maturation, protects

the embedded fluorophores for extended shelf life.

As shown in Fig. 4.3a, a fast initial release from freshly printed layers is observed (black lines). Flow experiments show that a 2-step gelatin maturation (green, blue and cyan lines) indeed results in a delay in antibody release. Initial maturation (2 days, 4°C, 85% RH) contributes a substantial part of the overall reduction in the fractional release during blood inflow. Ongoing physical crosslinking of gelatin during follow-up maturation (1 to 6 weeks, 4°C, 10% RH) further slows the release kinetics, and also the increasing fraction of antibody trapped in the layer can most likely be attributed to extensive gelatin crosslinking. Additionally, the delayed release kinetics from matured layers observed in the flow chambers is confirmed by the much reduced antibody wash-off in the counting chambers. Fig. 4.3b shows the profiles of the fluorescence intensities (APC- α CD3 readout) along the inflow direction of the filled counting chambers containing differently matured layers. Strong antibody wash-off in the counting chambers with freshly printed layers results in a severely inhomogeneous distribution of the local antibody concentrations, being ~ 25 times higher in the “venting region” of the chamber than in the “filling region”. Initial maturation largely prevents antibody wash-off and follow-up maturation further improves the uniformity of the distribution. Fig. 4.3c correlates data from flow and counting chambers: the fractional release during the inflow period ($M_{10\text{ s}}/M_{\text{total}}$) in the flow chambers and the background intensity ratio between the “venting and filling regions” in the counting chambers. It is apparent that the first 10 s of PBS flow give a good estimate of the effect of blood inflow on the antibody wash-off, at all stages of gelatin maturation.

Fig. 4.3: a) Fast initial APC- α CD3 release from freshly printed layers and delayed release from matured layers in the flow chambers after different conditions and periods of maturation. b) APC- α CD3 distribution in the counting chambers with freshly printed and differently matured layers after blood inflow. Dashed boxes denote the “filling region” and “venting region”. c) Correlation between fractional release during inflow (M_{10s}/M_{total} , black bars) and APC- α CD3 wash-off in the counting chambers (red squares). Data represents mean $\pm$ standard deviation ($n = 3$).

4.3.2 Temperature switched antibody release with on demand antibody-sample mixing

In order to trigger rapid and complete antibody release on demand, filled chambers were heated to 40°C (T switch). This actively controlled release was first characterized in the flow chambers. Fig. 4.4a clearly shows that, as the temperature of the flow chamber reaches the sol-gel transition temperature of gelatin (reported as ranging from 31 to 37°C)²⁹⁻³¹, a fast and complete antibody release is induced by the dissolution of the gelatin matrix (Fig. 4.S2). Fig. 4.4b compares the fractional antibody release of differently matured release layers after 10 min ($M_{10 \text{ min}}/M_{total}$). The employment of a T switch during incubation helps to free most of the trapped

antibody from matured layers, thus allowing for optimized immunostaining.

Fig. 4.4: a) Kinetics of APC- α CD3 release from differently matured layers with T switch. The dashed line represents the temperature of the flow chambers. Arrows indicate characteristic time points during release. b) Comparison of fractional release obtained after 10 min ($M_{10 \text{ min}}/M_{\text{total}}$) from differently matured layers incubated without (blue squares) and with (red circles) T switch. Data points represent mean $\pm$ standard deviation ($n = 3$).

The effect of temperature switched release was also examined in the counting chambers. It is noticeable in Fig. 4.5 that the T switch significantly reduces the inhomogeneity of the background intensity (i.e. retained print pattern) in matured layers, subject to both initial and follow-up (6 weeks) maturation.

Fig. 4.5: Fluorescence images (overlay of images with red (APC) and blue (PerCP) excitation light) of matured gelatin/antibody layers (subject to initial and 6-week follow-up maturation) after 40 min incubation with blood without T switch a) and with T switch b). The arrows indicate representative stained CD4⁺ T-lymphocytes (purple dots) and the rectangle marks a cell free region with clearly visible print pattern. Scale bars 0.5 mm. The displayed regions represent 9% of the complete image areas. (As can be seen also in the complete overlay image shown in Fig. 4.S3, cells distribute randomly throughout the complete image area of 7.8 mm $\times$ 5.2 mm.)

4.3.3 Optimized on-chip immunostaining

Finally, the homogeneity of cell intensities (after 40 min of incubation) was assessed by comparing the intensity of stained CD4⁺ T-lymphocytes located at the “filling” versus the “venting regions” of differently matured counting chambers. In chambers containing freshly printed layers, CD4⁺ T-lymphocytes identified at the “filling” versus the “venting regions” are clearly separated in the scatter plot of their fluorescence intensities (both APC- α CD3 and PerCP- α CD4 readout, Fig. 4.6a). Such inhomogeneous cell intensities are due to strong antibody wash-off. In contrast, layers subject to initial maturation already prevent antibody wash-off during blood inflow to some extent. The resulting antibody distribution with improved homogeneity contributed to more homogenous cell staining (Fig. 4.6b), which is indicated by the merging of the two cell populations identified in the “filling and venting regions”. As the storage proceeded to the follow-up stage (up to 6-week), extensively matured layers greatly prevented antibody wash-off and resulted in an optimized antibody distribution after blood inflow and uniform immunostaining (Fig. 4.6d). However, the intensities (both APC- α CD3 and PerCP- α CD4 readout) of cells are much below the levels of fully stained cells, which were determined previously to be very close to 10⁴ CPS in both channels.^{11, 23} The application of a *T* switch, after blood inflow, on matured layers causes complete antibody release and maintains the uniform antibody distribution, ensuring CD4⁺ T-lymphocytes are sufficiently stained and homogeneously distributed across the counting chambers (Fig. 4.6c and 4.6e). Homogeneous cell staining close to the saturation levels ensures a clear discrimination of stained cells from the background and between different cell populations. Fig. 4.7 summarizes the intensities (APC- α CD3 readout) of identified CD4⁺ T-lymphocytes at “filling” versus “venting regions” of the counting chambers containing printed layers at different stages of gelatin maturation, after 40 min incubation, with and without *T* switch.

Fig. 4.6: Scatter plots and histograms of APC (red excitation) and PerCP (blue excitation) emission intensities from fluorescence images of the counting chambers containing: a) Freshly printed layers; b), c) Layers subject to initial maturation; d), e) Layers subject to initial maturation and follow-up maturation for 6 weeks. Grey symbols are all identified events across the entire chamber. Green symbols are CD3⁺ and CD4⁺ cells (CD4⁺ T-lymphocytes) found in the “filling regions”. Orange symbols are CD4⁺ T-lymphocytes found in the “venting regions”.

Fig. 4.7: Average intensities (APC- α CD3 readout) of stained CD4⁺ T-lymphocytes at “filling and venting regions” from chambers containing different layers after 40 min incubation with and without T switch. For each layer, average intensities are obtained from 3 replicates of chambers incubated with blood samples from anonymous and healthy donors. Data points represent mean $\pm$ standard deviation ($n = 3$).

4.4 Conclusion

We have developed an inkjet printing method to release immunostaining agents on demand in our microfluidic cell counting chamber in order to achieve optimal cell staining for image cytometry. In a stopped-flow configuration, such as used here, two criteria for homogeneous on-chip sample preparation must be fulfilled: Firstly, during the inflow period of the sample filling the chamber, reagent release should be prevented in order to avoid the wash-off of reagent, which in turn would cause loss of reagent and inhomogeneous reagent distribution within the chamber. Secondly, after filling, rapid and complete release should be achieved on demand to attain fast, homogeneous cell staining across the image area.

We have achieved to delay the initial release from our ink jet-printed gelatin/antibody layers by optimizing a maturation procedure, which causes an increased level of physical crosslinking of the gelatin matrix, effectively slowing down

reagent release. A combination of a short maturation phase (2 days) under cold, humid conditions followed by longer periods (more than 1 week) of cold, dry maturation represents the optimal solution to meet the required homogeneous antibody distribution for our application.

Furthermore, we can release the immunostaining agents on demand, rapidly and completely, by employing a T switch, which causes the gelatin matrix to dissolve, thereby releasing the entirety of the embedded antibody. Not only does this allow for optimal cell staining, but also eliminates the background inhomogeneity caused by trapped antibody (print pattern).

This tunable antibody release (initial delay followed by a triggered complete release) offers a great solution to achieve on-chip immunostaining for stopped flow, image cytometry applications. In addition to immunostaining based cytometry, this method for on-chip controlled release can also be used in many other biological assays, requiring temporally or spatially controlled release for complex sample-reagent mixing schemes.

4.5 Supporting information

4.5.1 Development of release kinetics of APC- α CD3 and its stability under different maturation conditions

Fig. 4.S1: a) Fractional release of APC- α CD3 during the inflow period from layers matured at 4°C under 85% relative humidity (RH) (blue circles) and 10% RH (red squares) for varying storage periods. b) Normalized fluorescence intensity (APC- α CD3 readout) of dry layers matured at 4°C under 85% RH (blue circles) and 10% RH (red squares) for varying storage periods, respectively. Data points represent mean $\pm$ standard deviation (n = 3).

4.5.2 Temperature dependent layer integrity

Fig. 4.S2: The thickness ratio of layers, which are subject to initial maturation at 4°C under 85% RH for 2 days and follow-up maturation at 4°C under 10% RH for 6 weeks, before and after incubation in PBS at different temperatures for 3 min. Data points represent mean $\pm$ standard deviation (n = 3).

4.5.3 Distribution of stained cells in cell counting chambers

Fig. 4.S3: A complete overlay image (including both APC/red and PerCP/blue excitation) of matured gelatin/antibody layers (subject to 2-day initial and 6-week follow-up maturation) after 40 min incubation with blood. Randomly distributed stained cells are shown. Double positive cells are CD4⁺ T-lymphocytes, seen as purple dots.

4.5.4 Comparison of release kinetics between APC- α CD3 and PerCP- α CD4 from different printed layers

Fig. 4.S4: Kinetics of APC- α CD3 (red) and PerCP- α CD4 (blue) release from freshly printed layers (open circles) and from layers subject to 2-day maturation at 4°C under 85% RH (solid circles). The differences between the release kinetics of the two antibodies are negligible.

References

1. C. D. Chin, V. Linder and S. K. Sia, *Lab Chip*, 2012, 12, 2118-2134.
2. B. Weigl, G. Domingo, P. Labarre and J. Gerlach, *Lab Chip*, 2008, 8, 1999-2014.
3. P. Yager, T. Edwards, E. Fu, K. Helton, K. Nelson, M. R. Tam and B. H. Weigl, *Nature*, 2006, 442, 412-418.
4. X. Li, D. R. Ballerini and W. Shen, *Biomicrofluidics*, 2012, 6, 011301.
5. A. W. Martinez, S. T. Phillips, G. M. Whitesides and E. Carrilho, *Anal. Chem.*, 2010, 82, 3-10.
6. M. Hitzbleck and E. Delamarche, *Chem. Soc. Rev.*, 2013, 42, 8494-8516.
7. D. Erickson and D. Li, *Anal. Chim. Acta*, 2004, 507, 11-26.
8. M. Focke, D. Kosse, C. Muller, H. Reinecke, R. Zengerle and F. von Stetten, *Lab Chip*, 2010, 10, 1365-1386.
9. G. E. Fridley, H. Q. Le, E. Fu and P. Yager, *Lab Chip*, 2012, 12, 4321-4327.
10. K. E. Uhrich, S. M. Cannizzaro, R. S. Langer and K. M. Shakesheff, *Chem. Rev.*, 1999, 99, 3181-3198.
11. M. Beck, S. Brockhuis, N. van der Velde, C. Breukers, J. Greve and L. W. Terstappen, *Lab Chip*, 2012, 12, 167-173.
12. M. Mortato, L. Blasi, G. Barbarella, S. Argentiore and G. Gigli, *Biomicrofluidics*, 2012, 6, 044107.
13. E. Tekin, P. J. Smith and U. S. Schubert, *Soft Matter*, 2008, 4, 703-713.
14. M. Singh, H. M. Haverinen, P. Dhagat and G. E. Jabbour, *Adv. Mater.*, 2010, 22, 673-685.
15. P. Calvert, *Chem. Mater.*, 2001, 13, 3299-3205.
16. B. J. de Gans, P. C. Duineveld and U. S. Schubert, *Adv. Mater.*, 2004, 16, 203-213.
17. M. Tijero, R. Diez-Ahedo, F. Benito-Lopez, L. Basabe-Desmonts, V. Castro-Lopez and A. Valero, *Biomicrofluidics*, 2015, 9, 044124.
18. N. Komuro, S. Takaki, K. Suzuki and D. Citterio, *Anal. Bioanal. Chem.*, 2013, 405, 5785-5805.
19. W. Shen, M. Li, C. Ye, L. Jiang and Y. Song, *Lab Chip*, 2012, 12, 3089-3095.
20. J. Hou, H. Zhang, Q. Yang, M. Li, L. Jiang and Y. Song, *Small*, 2015, 11, 2738-2742.
21. F. Badii, C. Martinet, J. R. Mitchell and I. A. Farhat, *Food Hydrocolloids*, 2006, 20, 879-884.
22. M. Djabourov and P. Papon, *Polymer*, 1983, 24, 537-542.
23. X. Zhang, D. Wasserberg, C. Breukers, L. W. M. M. Terstappen and M. Beck, *Analyst*, 2016, 141, 3068-3076.
24. C. A. Schneider, W. S. Rasband and K. W. Eliceiri, *Nat. Methods*, 2012, 9, 671-675.
25. P. V. Kozlov and G. I. Burdygina, *Polymer*, 1983, 24, 651-666.
26. P. Godard, J. J. Biebuyck, M. Daumerie, H. Naveau and J. P. Mercier, *J. Polym. Sci., Part B: Polym. Phys.*, 1978, 16, 1817-1828.
27. I. Yakimets, N. Wellner, A. C. Smith, R. H. Wilson, I. Farhat and J. Mitchell, *Polymer*, 2005, 46, 12577-12585.
28. F. Badii, W. MacNaughtan and I. A. Farhat, *Int. J. Biol. Macromol.*, 2005, 36, 263-269.
29. O. Miyawaki, Y. Norimatsu, H. Kumagai, Y. Irimoto, H. Kumagai and H. Sakurai, *Biopolymers*, 2003, 70, 482-491.
30. N. G. Parker and M. J. W. Povey, *Food Hydrocolloids*, 2012, 26, 99-107.
31. S. Kawabe, M. Seki and H. Tabata, *J. Appl. Phys.*, 2014, 115, 143103.

Chapter 5

Automated fabrication of microfluidic CD4 counting chips containing staining agents using drop-on-demand printing methods

Dorothee Wasserberg, Xichen Zhang, Christian Breukers, Bridgette Connell, Ellen Baeten, Dorine van den Blink, Andries Bloem, Monique Nijhuis, Anne Wensing, Leon W. M. M. Terstappen, Markus Beck

Abstract

We demonstrate the fabrication of fully printed microfluidic CD4 counting chips with complete on-chip sample preparation and their applicability as a CD4 counting assay in research and clinical settings. CD4 counting in low-income and resource-limited point-of-care (POC) settings is only practical and affordable, if testing chips can be fabricated at very low cost and all manual sample preparation is avoided, while operation as well as quantification is fully automated and independent of the skills of the operator. Here, we show the successful use of (inkjet) printing methods to fabricate microfluidic cell counting chambers with controlled heights, and to deposit hydrogel matrix with embedded fluorophore-labeled antibodies for on-chip reagent storage and sample preparation. Studies of the release kinetics of antibodies from the hydrogel matrix revealed that inkjet printing changes the material properties of the hydrogel (gelatin) significantly. This necessitates the active control of reagent release on both key time scales, initially delayed release during sample inflow (10 s) and complete release (≤ 10 min) for saturated cell staining. Initial delay was achieved by adjusting sample preparation and maturation conditions, whereas an external stimulus (T-switch) was used to release the entirety of the embedded antibody for cell staining within two minutes, while at the same time homogenizing the fluorescence background.

We show the successful application of our fully printed CD4 counting chips on samples from healthy donors as well as on patient material and find a very good agreement between results from our method and the gold standard, flow cytometry in both cases.

5.1 Introduction

Despite the World Health Organization's (WHO's) and many countries' concerted efforts, there are still more than 2 million new human immunodeficiency virus (HIV) infections every year, adding to the more than 36 million people currently living with HIV, many of whom are not aware of their HIV status.¹ The extent of this pandemic, especially when considering that HIV prevalence is still the greatest in resource-limited sub-Saharan Africa, calls for very low-cost and simple POC (rapid) diagnostic tests to diagnose and stage HIV. HIV staging in POC settings is commonly done by means of CD4 counting, i.e. the enumeration of CD4 positive (CD4⁺) T-lymphocytes.

A large number of POC CD4 counting devices and techniques have been reported and reviewed recently.^{2,3} Reported examples, are based on a wide variety of principles, including cell counting assays based on optical detection⁴⁻¹³, label-free impedance measurements of cell lysates^{14,15}, or even simpler methods, like volumetric estimation of cell counts¹⁶ or lateral flow type assays¹⁷. Most devices fulfill many, but never all, of the WHO's ASSURED (Affordable, Sensitive, Specific, User-friendly, Rapid and robust, Equipment-free, Delivered) criteria¹⁸ for POC CD4 counting technology and to date, none of these commercially available POC assays has proven simple, cheap and reliable enough to be widely established.

We, therefore, see the greatest opportunity for improvement in simplifying sample preparation as well as reducing price per test (e.g. cost of disposals and reagents). In our opinion, a successful POC CD4 counting assay for resource-limited settings should fully integrate all sample preparation on a disposable testing chip without the need for manual or automated external intervention. Sample handling should be restricted to loading of the chips and not require any precision pipetting or specific skill sets, while at the same time minimizing the risks of excessive/extended handling of patient material. Furthermore, no cooling of perishable liquid reagents should be necessary, greatly reducing the volume and urgency for the logistics of the supply chain. The fabrication of the disposable (counting chambers and reagent materials) should be done using a low-cost method, also capable of high volume production.

On-chip sample preparation with integrated (dry) reagent storage on chip using diverse methods, has been reported on in the literature.¹⁹⁻²² A large variety of concepts are based on preserving (dried) reagents by embedding them in so-called matrix materials. Such matrix materials have the additional function of controlling reagent release. Many studies have been published on the successful storage and release of viable biomolecules from both, naturally and synthetically derived hydrogels off-chip^{23, 24}, while the preservation of viable biomolecules on-chip has been abundantly proven, either via encapsulation/embedding^{9, 25, 26} or covalent immobilisation^{10, 27-29}. Therefore, hydrogels are well-suited to act as matrix materials for the preservation and controlled release of biological reagents in microfluidic devices.³⁰⁻³²

Based on this concept, we previously reported on a very simple CD4 counting assay with complete on-chip sample preparation. In short, microfluidic cell counting chambers with defined chamber height are filled with finger prick blood samples by capillary forces without external flow control. All reagents (fluorophore-labeled antibodies) necessary for sample preparation (immunostaining) are already embedded in a dry hydrogel matrix (gelatin) coated in the counting chambers. The hydrogel matrix delays the release of staining agents until sample inflow is complete, thereby ensuring homogeneous antibody distribution throughout the entire chamber during incubation. The homogeneous distribution of staining agents on the mm-length scale in x- and y-direction of the chamber makes the release possible to solely rely on diffusion across z-direction, i.e. the chamber height (tens of μm), to mix sample and staining agents very quickly. After incubation, the chambers are imaged in a custom-built, wide field of view fluorescence imager, which also performs the automated image analysis, resulting in a CD4 count (number of CD4⁺ T-lymphocytes per μL). Quantification is done volumetrically exploiting the fact that the imaged volume (chamber height $\times$ image area) is known. However, this proof-of-principle still relied on labor-intensive manual chip manufacturing, making low-cost high-volume production impossible.

In this work, we report on the development of low-cost fabrication methods, with high-throughput capability to produce such POC CD4 counting chips, as described above.

For the construction of our cell counting chambers, we decided to use two standard microscopic substrates as substrate and capping slides, due to their availability and low-cost. A technique is needed, to attach these two slides to each other, while at the same time fabricating a sealed compartment, in which the sample can be confined. The required technique has to be simple and low-cost and make both, rapid prototyping and large volume production possible. Furthermore, the technique needs to provide a means to control chamber heights. A number of methods were considered: (screen/offset) printing of suspensions of spacer material in a sealing liquid, laminating adhesive, (UV-)lithography and injection molding. Lithography and injection molding rely on the expensive and time-/labor-intensive fabrication of masks or molds, many of those techniques are designed to replicate designs in large numbers using large amounts of fluid, which precludes rapid prototyping and flexibility in chamber design. Automating manual fabrication methods, like the one described above³² using laminating adhesive, with cut-outs forming microfluidic chambers, would require a custom-built assembly set-up to automate the process (e.g. roll-to-roll) and, moreover, specifications for consistent precision in height, cannot meet our requirements. We, therefore, chose drop-on-demand printing of glue/spacer mixtures for the fabrication of our chamber walls. Printing the outline of the compartments using glue/spacer mixtures defines the geometry of the counting chambers. The glue acts as the seal for these compartments, while the spacer material determines the height of the counting chambers. This method allows us to immediately start prototyping and make the rapid adjustment/optimization of chamber designs possible. Furthermore, printing techniques are suited for up-scaling and can therefore be used for large volume production. We chose microbeads of precisely controlled size as spacer material, which have been used in a wide range of fields for many different types of application.³³

The second component of the testing chips is the hydrogel matrix with embedded antibody. Numerous methods for the automated deposition of biomolecules exist and have been comprehensively reviewed by Hitzbleck et al.³⁴, who discuss, among others, commercially available systems for spray coating, inkjet printing and contact printing. For our application, we chose inkjet printing, because it is inherently suitable for rapid

prototyping and fast up-scaling, as well as patterning for future parallelization, e.g. in an array format. In addition, inkjet printing has been shown to be suitable for the deposition of macromolecules³⁵⁻³⁷ and at the same time to be biocompatible^{9, 26}.

In the following, we will discuss the key parameters we have optimized for the development of (inkjet) printing methods, used for the fabrication of fully printed CD4 counting chips and will prove their functionality by showing their successful application in research and clinical settings.

5.2 Experimental

5.2.1 Materials

All used water samples were of milliQ quality. All materials were used as received, unless stated otherwise. 20 μm diameter, monodisperse non-crosslinked poly(styrene) (PS) beads (TS 20) as well as 20 μm and 30 μm diameter, monodisperse crosslinked PS beads (Calibre™, CS 20 and CS 30) from Microbeads AS were suspended in UV-curable glue (Byllux 6308, viscosity: 40 mPa·s), purchased from Byla GmbH. Gelatin (type A, Bloom 295 g) was purchased from Sigma-Aldrich, trehalose from Merck. Fluorophore-labeled antibodies, allophycocyanin-labeled antiCD3 IgG (APC- α CD3, clone SK7, ~260 kDa) and peridinin-chlorophyll-labeled antiCD4 IgG (PerCP- α CD4, clone MEM241, ~308 kDa), were purchased from BD and Exbio, respectively. Gelatin and gelatin/antibody mixtures used as inks for printing were all filtered gravitationally prior to use, with a membrane filter (CellTrics®, mesh 20 μm , Partec). An antibody cocktail for flow cytometry (CD3/CD4/CD8/CD45 Multitest™), fluorescence counting beads (Trucount™ absolute counting tubes) and FACS™ Lysing Solution from BD were used, according to the manufacturer's specifications. Standard microscopy glass slides (76 mm $\times$ 26 mm $\times$ 1 mm) from Menzel and lasercut plastic slides made from poly(methyl methacrylate), TOPAS cyclic olefin copolymer, ZEONEX cyclic olefin polymer and poly(styrene) (PMMA, COC, COP and PS, respectively, 70-76 mm $\times$ 26 mm $\times$ 1 mm) were used. Prior to use, glass slides were rinsed with copious amounts of water, ethanol and acetone, then dried under a stream of N₂, while plastic slides were rinsed with copious amounts of isopropanol and dried under a stream of N₂. Blood samples for testing and calibration were obtained anonymously from healthy

individuals at the University of Twente, all of whom had provided written informed consent in advance. With respect to the use of patient materials for clinical study, the Medical Research Involving Human Subjects Act in the Netherlands (WMO) does not apply to the current study (the study of patient material using image cytometry as described here) and therefore an official approval of this study by the Medical Ethical Testing Commission at the University Medical Centre Utrecht (UMCU) was not required under the WMO. 105 samples from HIV positive patients who had given written informed consent for the use of left-over blood samples for anonymous research purposes were provided. Left-over blood samples, from which CD4 counts had been routinely determined using a BD FACS Canto II v3.0 at UMCU, were used to study the performance of the image cytometry method vs. the standard flow cytometry. Unless stated otherwise, all blood samples contain EDTA as anticoagulant. Calcium chloride (10 mM final concentration) and thrombin (0.6 units mL⁻¹ final concentration) were added to those samples immediately before use.

5.2.2 Equipment

A custom-built laser interferometer was used to determine chamber heights (manuscript in preparation). Briefly, the light from an edge-emitting laser diode ($\lambda = 405$ nm, beam divergence FWHM $\sim 20^\circ$) is collimated and focused at a single point of a counting chamber with two high numerical aperture lenses (diameter = focal length = 25.4 mm). The transmitted light is collimated by another lens of the same type, filtered by neutral density filters and falls onto the CCD chip of a camera. Since part of the light is reflected on the inner surfaces of the chamber, the resulting path length difference between the beams results in constructive (path difference = $n \times \lambda$) or destructive (path difference = $(n + 1/2) \times \lambda$) interference, depending on the transmission angle (θ), which corresponds to different positions on the camera. The resulting interferogram is analyzed by plotting the intensity against $\cos(\theta)$, resulting in a periodic intensity profile, the periodicity of which, determined by Fourier analysis, yields the chamber height.

The topography of gelatin/antibody layers was determined using a white light interferometer (smartWLI-microscope, GBS, height resolution ~ 10 nm).

Fluorescence images for image cytometry and layer characterization were

recorded using a custom-built fluorescence imaging system³⁸ with wide field of view (7.8 mm × 5.2 mm) and low optical resolution ~10 μm. A portable version of the above fluorescence imager (field of view: 8.9 mm × 6.7 mm), based on the same principles with built-in, automated image analysis, was used during the clinical study.

Reference values for CD4 counts from healthy donors were determined using a BD FACS ARIA 2, according to manufacturer specified protocol.

The (fluorescence) microscope, used for both fluorescence and bright field imaging, was a Nikon Eclipse E400 equipped with a 100 W mercury arc lamp, a Hamamatsu C4742-95-12NRB CCD Camera and 4× and 10× Nikon objectives. Appropriate filter sets were purchased from Semrock.

5.2.3 Chamber fabrication by piezo-actuated deposition

Ink preparation: 1% w/v suspensions of microbeads in UV-curable glue were prepared freshly, each day, by re-suspending aliquots of previously washed and dried microbeads.

Dispensing: For the deposition of chamber walls, a Pipejet P9 nanodispenser (Biofluidix GmbH, Germany) with a 200 μm orifice was used with the following settings (Fig. 5.S1): 200 μm ms⁻¹ down stroke velocity, 10 μs hold time and 30 μm stroke length.^{39, 40}

Assembly: A custom-built setup was used to automatically dispense the glue/bead suspension as well as assemble and cure counting chambers on microfluidic chips. The nanodispenser was mounted on a L-60 Movtec xyz-mobile gantry to dispense the glue/bead suspension to form the outlines of the later chamber walls. Immediately after dispensing the curable glue/bead suspension on a substrate plastic slide, the grabber unit of the setup was used to automatically retrieve a capping glass slide with glass weight, place it on the freshly printed substrate slide, leave it to settle for 5 s and cure the glue using a 405 nm Luminus CBT-90UV LED at 5 A for 1 s. For further discussions of the chamber fabrication process and parameters see below.

5.2.4 Gelatin/antibody deposition by inkjet printing

Ink preparation: To prepare solutions for inkjet printing, gelatin powder was dissolved in water at 40°C for 1 h while stirring vigorously. To the resulting solution, 0.6 μg mL⁻¹

APC- α CD3, 0.2 $\mu\text{g mL}^{-1}$ PerCP- α CD4 stock solutions and water were added to yield final concentrations of 0.3% w/v of gelatin, 10 ng mL^{-1} APC- α CD3 and 3 ng mL^{-1} PerCP- α CD4. The solutions were left to cool to room temperature (RT), and filtered before use.

Inkjet printing: Inkjet printing was performed using an industrial inkjet printer (LP50, PixDro, Meyer Burger B.V.) equipped with a Konica Minolta, 512MHX printhead with 512 nozzles in 2 staggered rows. Droplets of ~ 20 pL were dispensed in multiple swaths at $(360 \text{ dpi})^2$ resolution (“native resolution”), resulting in final resolutions between $(360 \text{ dpi})^2$ and $(1800 \text{ dpi})^2$. Unless stated otherwise, printed reagent layers were stored at 4°C and $\sim 10\%$ relative humidity (RH). For further discussions of the fabrication process, see below.

5.2.5 Leukocyte depletion and leukocyte spiking

Blood samples with a large range of CD4 counts were prepared to determine the correlation between CD4 counts obtained using our image cytometry method with those obtained using the reference method, flow cytometry, which is considered the gold standard. First of all, whole blood was centrifuged at $1000\times g$ for 15 min to separate blood plasma, leukocytes (buffy coat) and erythrocytes. After first removing most of the plasma, the buffy coat was carefully aspirated and then, an erythrocyte fraction was collected. Subsequently, plasma and erythrocyte fractions were recombined, at a ratio of 1:1, to mimic the natural hematocrit, yielding a leukocyte depleted sample. The CD4 counts of leukocyte depleted and buffy coat samples were determined using flow cytometry ($n = 3$). The resulting CD4⁺ T-lymphocytes concentrations of the leukocyte depleted and the buffy coat samples, were used to calculate mixing ratios for those two fractions resulting in a number of samples with calculated CD4 counts in the range of $5 \mu\text{L}^{-1}$ to $1400 \mu\text{L}^{-1}$.

5.3 Results and discussion

5.3.1 Fabrication of counting chambers by printing

As discussed in the Introduction, we chose drop-on-demand printing of UV-curable glue containing microbeads to fabricate our counting chambers. Glue/bead mixture

was dispensed onto a substrate along the outline of the intended chamber walls and then a glass slide was added as cover (Fig. 5.1 and 5.2). The UV-curable glue was dispensed in an uninterrupted line to ensure the restriction of sample fluid to the assembled chamber after curing. Chamber heights were precisely controlled by suspending mono-sized microbeads with a diameter of the intended chamber height ($\sim 30\ \mu\text{m}$) in the UV-curable glue, ensuring a defined distance between top and bottom of the assembled chamber.

Fig. 5.1: Schematic representations of the chip design (top) and zoom-in (middle). The fluorescence image of a dry layer is in the left chamber; fluorophore-stained cells are shown as overlays of the fluorescence images of both fluorophores in the right chamber. Topography (bottom, left) and fluorescence image (bottom, center) of the same segment of an printed layer. Bright field image (bottom, right) of a part of a printed and cured chamber wall. White arrow points at a single microbead.

Materials for the fabrication of chambers were selected based on the following criteria: reproducibility of dispensing ink, compatibility of microbeads and glue, compatibility of glue and substrate, and control over chamber heights.

We achieved reliable drop dispensing of Byllux 6308 UV-curable glue, resulting in drop volumes of ~ 8 nL. Drop dispensing showed excellent reproducibility chip-to-chip as well as day-to-day.⁴¹ Suspensions of crosslinked PS microbeads in Byllux 6308 UV-curable glue were found to be stable overnight (Fig. 5.S2). The sedimentation of the beads in the glue was avoided by matching the densities of the two materials (1.05 g cm^{-3}). PMMA was chosen as a substrate material to ensure that printed features would remain stable on the substrate (no de-wetting, Fig. 5.S3). A printing pattern was designed to ensure the coalescing of the dispensed drops into stable printed features. To this end, the drop-to-drop distance and the geometric pattern of the dispensed drops were optimized for good reproducibility of uninterrupted printed features. Fig. 5.S4 shows cured lines, printed using two different geometric patterns (linear and 45° zig-zag) and various drop-to-drop distances (0.2-0.6 mm). Clearly, features printed using the linear pattern did not coalesce, independent of the drop-to-drop distance, while all features printed using zig-zag patterns coalesced into stable features. Therefore, zig-zag patterns with an intermediate drop-to-drop distance of 0.5 mm were chosen for the fabrication.

The feasibility of controlling the heights of our printed counting chambers was verified. The heights d of a series of 182 consecutively printed chambers were determined, using laser interferometry, to be on average $31.6 \pm 3.5 \text{ }\mu\text{m}$. After inspection, 17 chambers were excluded, that seemed to contain trapped particles of diameters larger than $30 \text{ }\mu\text{m}$. The average height d of the remaining 165 chambers was determined to be $30.7 \pm 1.2 \text{ }\mu\text{m}$.

Fig. 5.2: Bright field images of a single drop of cured Byllux 6308 glue containing Calibre CS 30 microbeads (30 μm diameter) on PMMA substrate covered by glass slides (top, left), Calibre CS 30 microbeads in the cured glue (bottom, left), a printed and cured line of glue/bead suspension being part of an assembled chamber (center). White arrows indicate individual microbeads. A photograph of 3 consecutively fabricated chips before (top, right) and after (bottom, right) filling.

5.3.2 Inkjet printing of hydrogel/reagent layers

We employed inkjet printing for the fabrication of a thin layer of gelatin as matrix, in which the staining agents were embedded. The gelatin matrix has two functions: First, to minimize the degradation of staining agents, i.e. fluorophore-labeled antibodies, during storage in the (dried) layers. Second, to ensure homogeneous cell staining throughout the chamber.

First, the feasibility of storing fluorophore-labeled antibodies in a gelatin matrix will be addressed. As can clearly be seen in Fig. 5.S5, dry gelatin/antibody layers, stored under different conditions, show a noticeable drop of fluorescence intensity to $\sim 80\%$ after < 100 days. An even larger reduction of cell staining intensities after < 100 days to about 40-50% is also evident. This probably means that both fluorophore integrity as well as antibody viability are decreasing in time, jointly causing the reduced cell staining intensity. Due to the above results, any CD4 counting chip shown here, had been stored less than 8 weeks (56 days) prior to use.

The second function of the gelatin matrix is to control the timing of the release of staining agents into the cell counting chamber. On one hand, the release of staining agents must be delayed, until sample inflow has completed (stopped-flow configuration), which takes on the order of ~ 10 s. If the delay is too short, a significant fraction of antibody will be released during sample inflow and, subsequently, be washed toward the chamber exit, causing substantial inhomogeneity in cell staining. On the other hand, the entirety of the embedded antibody must be released fast enough (< 10 min) to make rapid staining and testing within 30 min feasible, while background inhomogeneity, due to trapped antibody, is kept as low as possible.

Gelatin is well suited as a release matrix to fulfill these two conditions. Dry gelatin/antibody layers require some time to take up enough water (from the inflowing sample) to reach sufficient swelling (equilibrium swelling ratio $\sim 14\times$), i.e. sufficient pore size, and until then can the embedded antibody diffuse out of the matrix. This delay in antibody release has previously been determined to take place on the order of tens to hundreds of seconds for a few hundred nm thick layers.⁴² Furthermore, once swollen, gelatin layers are capable of the rapid release of the majority of embedded antibody, well within the first 10 min after sample inflow. Kinetic data to that effect has been published elsewhere.⁴²

In order to translate our earlier results, gathered on cast samples⁴², to printed gelatin/antibody layers, the first step was the development of a method to print layers that are as homogeneous as possible, with regard to both topography as well as fluorescence intensity. Obviously, homogeneous staining can best be achieved above a topographically homogeneous release layer, especially in a stopped-flow configuration. Additionally, the more homogeneous the distribution of antibody in the layer is, the higher the signal to noise ratio, improving sensitivity.

As a first step, the topographical homogeneity of printed pure gelatin layers was optimized. The following conditions were found to be essential to ensure reproducible and stable jetting of aqueous gelatin solutions, using a Pixdro LP50 and a Konica Minolta KM512 printhead: The concentration of gelatin was kept constant at 0.3% w/v. With regard to printhead, the KM512MH with intermediate nozzle diameter (~ 27 μm) was selected as compromise between maximized resolution and minimized shear

forces, see below. Furthermore, the clogging of nozzles due to drying had to be prevented by, first, always using the native x-resolution of the printhead (360 dpi), i.e. always using all 512 nozzles; second, keeping the print speed $\geq 5 \text{ mm s}^{-1}$ and third, avoiding active heating of the substrate and printhead. Complying with the aforementioned prerequisite settings, we set out to develop an inkjet printing method for the fabrication of (pure) gelatin layers with optimized topographical homogeneity.

In previous work, we analyzed kinetic release data and found that the results fit well with a diffusion-controlled release model.⁴² This means that antibody release is strongly dependent on the thickness of the gelatin matrix. Therefore, we used our earlier results as a starting point for a projected thickness of gelatin/antibody layers in the range of few hundred nm. Layers of such thicknesses represented a compromise between sufficient initial delay of release and complete release ($\leq 10 \text{ min}$) within the incubation time of 30 min. It should be mentioned here that the released fraction of antibody on both time scales (10 s and 10 min) also depend strongly on the conditions under which the layers are formed and left to mature⁴³, which will be discussed in greater detail below.

We have found that, in order to fabricate topographically homogeneous, i.e. smooth, gelatin layers, the key parameter that needs to be optimized is the amount of dispensed gelatin solution per unit time, i.e. the dispensing rate. If too much solution is dispensed per unit time, there will not be sufficient time for water to evaporate, before the upcoming liquid from the next pass (swath) of the printhead. Eventually, the accumulation of solution will reach a point when it will cause a redistribution (“puddle formation”) of material and, thus, topographical inhomogeneity on large (mm to cm) length scales.

The principal printing parameters that were varied, to determining the optimal dispensing rate, were print speed and final resolution of the layer. All other parameters influencing the amount of liquid dispensed per unit time were predetermined by the need to ensure stable jetting as mentioned above, like the resolution per swath and the concentration of gelatin. Similarly, a temperature increase to speed up drying, thus allowing for larger dispensing rates, was not compatible with the requirement of achieving stable jetting.

The print speed was varied starting from the lowest possible value of 5 mm s^{-1} to 15 mm s^{-1} and 50 mm s^{-1} . As can be seen in Fig. 5.S6, the higher the print speed is, the less homogeneous the printed layers are. Redistribution of large amounts of material, on the length scale of several millimeters, seems to have taken place in layers printed using 15 mm s^{-1} and 50 mm s^{-1} , causing the formation of “puddles”. The length scale of the redistribution seems to be largest for layers printed using 50 mm s^{-1} , while the topographical inhomogeneity of layers printed using a print speed of 5 mm s^{-1} , appears on a length scale much smaller than one mm. We therefore used a standard print speed of 5 mm s^{-1} for all further printing of gelatin/antibody layers.

Next, layers were fabricated with final resolutions of $(360 \text{ dpi})^2$, $(1080 \text{ dpi})^2$, $(1440 \text{ dpi})^2$ and $(1800 \text{ dpi})^2$ in a single print job. The final resolution of the printed layer essentially changes the amount of dispensed material and should therefore be directly correlated to the layer thickness. As Fig. 5.3 clearly shows, we are well able to control the thickness of printed gelatin layers by varying their final resolution. However, it is also evident from results shown in Fig. 5.3 that roughness, represented by the (averaged) standard deviations (SDs) of the mean layer thicknesses, increases with layer thickness. This means that for best homogeneity, the layer thickness should be minimized, where possible.

Fig. 5.3: Representative white light interferometry images of (single print job) gelatin layers printed at resolutions of $(360 \text{ dpi})^2$ (top, left), $(1080 \text{ dpi})^2$ (top, right), $(1440 \text{ dpi})^2$ (center, left) and $(1800 \text{ dpi})^2$ (center, right). Calibration bars are roughly centered on the average layer thicknesses (nm scale, $n \geq 6$). Images represent an area of $0.71 \text{ mm} \times 0.54 \text{ mm}$. A plot with the averaged layer thicknesses determined from white light interferometry images (bottom, left) and the same data plotted against the number of dispensed drops (bottom, right). Data points represent mean thickness $\pm$ standard deviation of exemplenary layers prepared in different conditions.

In order to improve topographical homogeneity, printing gelatin layers in multiple print jobs was tested. The reasoning is as follows: A break ($\geq 1 \text{ min}$) between

print jobs would allow for additional drying time, which is expected to improve smoothness. A layer with a final resolution of $(1440 \text{ dpi})^2$ was selected to test this hypothesis: The topography of layers printed in a single print job ($1 \times (1440 \text{ dpi})^2$) were compared (Fig. 5.3) with those of layers printed in multiple print jobs ($4 \times (720 \text{ dpi})^2$ and $18 \times (360 \text{ dpi})^2$). Clearly, dividing layer printing into a number of separate print jobs results in no marked improvement of the layer smoothness. Single print job was, thus, adopted as a standard printing strategy, as it makes fabrication faster with a higher chance of maintaining stable jetting, without loss of layer quality.

Having arrived at an optimized method to produce layers of highest attainable topographical homogeneity, we set out to fabricate gelatin/antibody layers to optimize antibody release and, thus, cell staining.

HIV-staging assays generally report the CD4 count. Therefore, the following fluorophore-labeled antibodies were selected as staining agents: APC- α CD3 (stains all T-lymphocytes) and PerCP- α CD4 (stains the CD4⁺ T-lymphocytes and, weakly, monocytes), which yields the required CD4⁺ T-lymphocytes as the double positive subpopulation after staining. The concentration of double positive cells, detected via image cytometry (divided by imaged volume), yields the CD4 count.

5.3.3 Optimization of reagent release from printed hydrogel layers

In previous work, we determined an optimal concentration to approach saturation of cell staining intensity (within 30 min) of a few times 10^8 antibody molecules per mm^2 for both APC- α CD3 and PerCP- α CD4, corresponding to antibody concentration in the counting chamber in the 10 nM range, consistent with equilibrium dissociation constants in the range of 1 nM.^{38, 42} Therefore, all gelatin/antibody layers were fabricated, aiming at an antibody density of 10^8 antibodies per mm^2 on the surface area of the counting chamber.

As mentioned above, the second function of the gelatin matrix is to ensure homogeneous cell staining. The following section will address the strategy we devised to achieve said homogeneous cell staining in detail. As discussed before, achieving homogeneous antibody release requires optimizations on two time scales: the delay of initial release during inflow as well as the complete release well within the incubation time. For our previously cast layers, a simple optimization of layer thickness provided

a solution to both problems, i.e. an optimal layer thickness could be found for which the release on both time scales (10 s and 10 min) could satisfy the criteria for homogeneous, saturated cell staining. However, analysis of the kinetics of antibody release from printed gelatin layers, revealed drastic changes, when compared with release from cast layers. In Fig. 5.4, kinetic release data is plotted against the square root of time. Samples of gelatin/antibody solution that had been passed through the printhead under different conditions (different power settings used for piezo actuation and/or different pressures) were all cast and their antibody release was recorded in time. As reference, stock solution was cast without having been passed through the printhead. Clearly, the initial phase of the release follows the square root dependence, which is expected for Fickian diffusion from a thin slab^{44, 45}:

$$\frac{M_t}{M_{\text{total}}} = \left(\frac{t}{\tau}\right)^{1/2} \quad (1)$$

where M_t is the mass released at time t , M_{total} is the total mass of antibody embedded in the layer. The process can be characterized by the release time τ , given by⁴⁶

$$\tau = \frac{\pi L^2}{4D} \quad (2)$$

where L is the thickness of the layer and D is the diffusion constant. It seems that increased shear exerted on the gelatin/antibody solution, while passing through the nozzle, either via increased constant pressure or pressure pulses induced by piezo actuation, results in faster antibody release from the resulting layer (up to a factor 3 faster).

This effect can be rationalized when considering that gelatin can undergo two types of physical crosslinking. Apart from the general physical crosslinks, which any sufficiently long macromolecule can form at sufficiently high concentrations, gelatin can form an additional type of supramolecular linkage: the re-forming of partial collagen-like triple helices.⁴⁷⁻⁴⁹ It is well established that the process of crosslinking via the formation of helical structures is slow (time scales in the range of days) and that the process very much depends on ambient conditions, e.g. RH and temperature.⁵⁰⁻⁵² With this in mind, one can interpret the effect of shear forces on gelatin solutions, as causing rupture of existing helical crosslinks, which are too slow

to re-form, in the time it takes for the sample dry. The resulting layers would then contain mostly the standard, so-called “random coil” type of crosslinks, effectively increasing the mesh size of the layers, which in turn would lead to faster antibody diffusion and, thus, faster release.^{42, 43}

Fig. 5.4: Kinetics of APC- α CD3 release (top, selected conditions) with fits to their initial release in order to determine release times, τ , and release times, τ (bottom), determined by measuring the release of APC- α CD3 in time, from layers cast using solutions passed through inkjet printing nozzles under the influence of shear forces of varying magnitudes (bottom, inset). As control, a solution was cast without having passed through a nozzle. Data points represent mean $\pm$ standard deviation ($n = 3$).

The changes of release times, τ , are even more pronounced, when directly comparing release from layers prepared by casting vs. printing (Fig. 5.5). The release

time, τ , of antibody release from printed layers is 7 times smaller, than that of cast layers of comparable thicknesses. This additional impact is probably due to the shorter drying times when comparing printed (< 90 s per swath) and cast samples (~ 20 min), supposedly preventing the slow formation of helical crosslinks even more effectively. Clearly, printed gelatin layers would have to be much thicker, than their cast counterparts, to achieve similar delays of initial antibody release. As has been previously determined, for a sufficient delay of initial release, a minimum release time of 100 s is required.⁴² However, dry printed layers would have to be more than $1.5\ \mu\text{m}$ thick on average to achieve $\tau \geq 100$ s, which would partially block the cell counting chamber upon swelling of the layer due to layer inhomogeneity.

Therefore, we used a method we devised earlier⁴³, to actively control the release on both time scales; the delay during the initial phase of release as well as the complete release during incubation. First, layers were exposed to increased RH (85%) in the cold (4°C) for two days, then left to mature further, under dry, cold conditions (Fig. 5.6). This treatment has been shown to induce efficient formation of helical crosslinks in gelatin layers.⁴³ These measures were highly effective in delaying initial antibody release (Fig. 5.6, open symbols) and making printed, then actively crosslinked gelatin/antibody layers, excellent candidates to realize initial release delay in our CD4 counting chips. Second, complete release during incubation was also actively controlled, by means of “switching” the state of the swollen gelatin layers from gel to solution, by increasing the temperature of the chip to 40°C . This T -switch allowed for the rapid and complete release of all remaining antibody in a layer, controlled by an external stimulus (Fig. 5.6, solid symbols). Such measures for controlled antibody release were proven to be beneficial in the cell staining application. Fig. 5.S7 demonstrates the effect of layer maturation and T -switch on homogenizing the fluorescence background in the cell counting chambers.

Fig. 5.5: Kinetics of APC- α CD3 release from cast (black symbols) and printed (red symbols) gelatin layers (top). Dependence of release times, τ , of APC- α CD3 on the average thickness (in the dry state) of cast (black symbols) and printed (red symbols) gelatin layers (bottom). Data points represent mean $\pm$ standard deviation ($n = 3$).

Fig. 5.6: Kinetics of APC- α CD3 release from differently matured layers with (solid symbols) and without (open symbols) T -switch. The dashed line represents the chamber temperature. Arrows indicate characteristic time points during release. Partially reproduced from ref. 56, copyright 2016 American Chemistry Society.

In the previous sections, we have shown that we have devised printing methods, to not only fabricate counting chambers of defined heights, but also to deposit release layers for on-chip storage and controlled antibody release on our CD4 counting chips. In the following section, the performance of such fully printed CD4 counting chips will be assessed.

5.3.4 Performance of fully printed CD4 counting chambers

The correlation of CD4 counts obtained using our image cytometry method, with CD4 counts obtained using flow cytometry was assessed. To this end, blood samples from healthy donors with a large range of different CD4 counts were used. A brief image processing workflow is shown in Fig. 5.7 (left panel). The resulting scatter plot (Fig. 5.7 right panel top) from image analysis yields the CD4 count. A variety of acquired CD4 counts are plotted against the result obtained by flow cytometry (Fig. 5.7 right panel bottom).

Fig. 5.7: Left panel, processing workflow of overlays of fluorescence images recorded under blue and red excitation: top, raw overlay image; center, overlay image after background correction; bottom, overlay image with ROIs marked by green circle. Scale bar 0.5 mm. Right panel: top, scatter plots and histograms of the blue vs. the red fluorescence intensities of all identified objects; bottom, comparison of average CD4 counts obtained from leukocyte-depleted and -spiked samples from healthy donors containing a large range of different leukocyte counts using image and flow cytometry. Data points represent means $\pm$ standard deviations ($n \geq 6$).

Clearly, the correlation between image and flow cytometry data is excellent for CD4 counts below 1000/ μL . In a CD4 counting application only counts $\leq 500/\mu\text{l}$ are clinically relevant, as this is the new threshold, below which antiretroviral therapy should commence.⁵³ Very high CD4 counts would call for

the modification of our CD4 counting chips (e.g. reduction of chamber heights), as the large number of stained cells make the automatic identification of separate objects difficult. We, therefore, excluded counts $\geq 1000/\mu\text{l}$ from fitting. Having shown the good correlation between methods, we proceeded to analyse a set of patient samples and compared our results with reference data obtained using flow cytometry.

Fig. 5.8 shows the outcome of the patient study. A good agreement between flow cytometry and image cytometry can be seen for many of the samples. However, cell counts from chambers stored more than 8 weeks are generally too low, which is mainly due to the reduced staining intensity. The degradation of fluorophores and insufficient antibody release as a consequence of excessive gelatin crosslinking in the aged counting chambers account for the poor immunostaining. In addition, some results obtained with chambers stored 8 weeks or less deviate considerably from the flow cytometry reference. This discrepancy is caused by heat-induced movement of the sample between the exposure with red and blue excitation light, which either results in the detection two single positive cells instead of one double positive cell or in streaks, if the movement is large during the exposure time. Such streaks are generally disregarded entirely, as the intensity per pixel falls below the set threshold and the roundness criterion is not met. Both cases will result in an underestimation of the CD4 count, which is clearly seen by the trend in the scatter plot of Fig. 5.8 as most dots (green and red circles) fall below the ideal correlation (black solid line).

It should be mentioned that calcium and thrombin were added to the samples immediately before filling the chambers in order to counteract the anticoagulant EDTA. However, the desired effect of suppressing cell movement was not sufficient in all regions of all chambers. A POC CD4 count will use finger prick blood without anticoagulants, and therefore naturally occurring blood clotting will prevent cell movement without the addition of calcium and thrombin.

Fig. 5.8: Comparison of CD4 counts obtained from patient blood samples using standard flow cytometry and image cytometry.

To address the problems of reduced cell intensity, images obtained from the counting chambers stored more than 8 weeks were excluded. With respect to the images of moving cells, an algorithm for the automated recognition of areas with moving cells in the imaging analysis has been implemented to reduce this problem. This algorithm makes use of the fact that the unstained cells, mainly erythrocytes, cause inhomogeneity in the fluorescence background from unbound antibody. Areas without cell movement are identified as regions where the background in the fluorescence images with red and blue excitation light correlate well. If this accounts for less than 50% of the image area, the test is excluded to ensure that a sufficient volume of sample is analyzed. The reanalysis of the remaining sample images resulted in a greatly improved correlation of CD4 count between standard flow cytometry and image cytometry (Fig. 5.9).

Fig. 5.9: Reanalysis of the images from the patient study, excluding chambers that were stored longer than 8 weeks and using automated recognition of areas with cells moving between the exposures with red and blue excitation light. These areas are excluded from the image analysis and if more than 50% of the image analysis has to be excluded, the complete chamber is excluded from the analysis.

5.4 Conclusion

In summary, we have demonstrated the fabrication of fully printed microfluidic CD4 counting chips with complete on-chip sample preparation and successfully demonstrated their applicability as a CD4 counting assay using blood from patients in need for CD4 count assessment.

We believe that the practical and affordable CD4 counting method in low-income and resource-limited settings should be cheaply fabricated and automatically operated from sample preparation to result analysis. Such a CD4 counting method can eliminate the need for expensive external equipment (disposables, pipettes, pumps etc.) and materials (reagent/lysing solutions, buffers) for sample preparation, while also precluding the reliance on trained personnel.

We started to develop such a method, and had shown previously, the development

of an automated CD4 counting assay, with the entirety of the sample preparation taking place on-chip, without any manual intervention and assistance by external equipment, with the exception of our image cytometer for fluorescence readout and automated analysis. However, this proof-of-principle still relied on labor-intensive manual methods for chip manufacturing and was, therefore, incompatible with low-cost fabrication. In the present work, we have shown the extension of our former research to low-cost fabrication of our CD4 counting chips via (inkjet) printing techniques.

We developed printing methods to, on one hand, fabricate chamber walls by dispensing mono-sized microbeads, suspended in UV-curable glue, using a piezo-actuated nanoliter dispenser. On the other hand, we developed an inkjet printing method to deposit hydrogel layers with embedded fluorophore-labeled antibodies, onto our microfluidic chips, for on-chip reagent storage and sample preparation. By employing optimized printing parameters, we achieved to fabricate counting chambers with well defined chamber height ($30.7 \pm 1.2 \mu\text{m}$) and deposited homogeneous gelatin/antibody layers in such chambers. In addition to printing, we executed extra measure (layer maturation and *T*-switch) to precisely control antibody release (initially delayed release + temperature triggered rapid dissolution) from printed layers for complete on-chip cell staining.

We successfully tested our CD4 counting chips on different healthy donors and found a good agreement between CD4 counts determined using our image cytometry method and using the gold standard, flow cytometry. We then proceeded to test the assay using left-over patient blood and again found a good agreement between results from our method and the standard after excluding aged counting chambers and poor quality images.

We are presently investigating, how to translate the methods, described here, to other essential POC cell counting applications, e.g. quantitative malaria detection. Preliminary data suggests that a translation to many other cell counting assays that rely on cell staining (via fluorophore-labeled antibody or nuclear dyes) is feasible. The concept gains great flexibility and speed via the use of printing techniques for fabrication and can, therefore, be adapted to rapidly test new cell counting assays.

5.5 Supporting Information

Fig. 5.S1: Schematic representation of the dispensing cycle of the nanodispenser³⁹.

Fig. 5.S2: Bright field microscopy images of crosslinked (top) and non-crosslinked (bottom) PS microbeads of 20 μm diameter suspended in Byllux 6308 UV-curable glue for 5 min (left) and for 16 h (right) prior to curing.

Fig. 5.S3: Bright field microscopy images of Byllux 6308 after printing on COC (top, left), COP (top, right), PMMA (bottom, left) and PS (bottom, right). Images each represent an area of $135 \mu\text{m} \times 178 \mu\text{m}$.

Fig. 5.S4: Schematic representations (left) and bright field microscopy images (right) of linear (top) and 45° zig-zag (bottom) print patterns at drop-to-drop distances varying between 0.2 mm and 0.6 mm.

Fig. 5.S5: Scatter plots of logarithmic cell staining intensities (PerCP- α CD4, blue vs. APC- α CD3, red) obtained from CD4 counting chips with gelatin/antibody layers, stored for 1 day (top, left), 26 days (top, center) and 97 days (top, right) as well as two plots detailing fluorophore and antibody degradation in time (bottom): fluorescence intensities of dry layers (bottom, left) and stained cells (bottom, right) after storage up to 90 days. Data points represent mean $\pm$ standard deviations ($n = 3$).

Fig. 5.S6: Stitched bright field images of gelatin layers printed at print speeds of 5 mm s⁻¹ (left, standard), 15 mm s⁻¹ (center), 50 mm s⁻¹ (right), while otherwise, standard parameters and conditions were applied. Scale bar 2.1 mm.

Fig. 5.S7: Effect of layer maturation and *T*-switch on the distribution of antibody in the counting chamber. Overlays of fluorescence images with blue (PerCP) and red (APC) excitation of a freshly printed layer (top, left) and a layer subject to initial and 6-week follow-up maturation (bottom, left) after blood inflow. Blood flows from the left end of the image. Scale bar 1 mm. Zoom-in overlay of fluorescence images of matured layers subject to initial and 6-week follow-up maturation after 40 min incubation with blood without *T* switch (top, right) and with *T* switch (bottom, right). Scale bar 0.5 mm. Partially reproduced from ref. 56, copyright 2016 American Chemistry Society.

References

1. UNAIDS, <http://aidsinfo.unaids.org/>
2. M. T. Glynn, D. J. Kinahan and J. Ducree, *Lab Chip*, 2013, 13, 2731-2748.
3. G. L. Damhorst, N. N. Watkins and R. Bashir, *IEEE. Trans. Biomed. Eng.*, 2013, 60, 715-726.
4. Partec GmbH, Cyflow[®] Minipoc Product Information Document, 2011
5. Alere, Pima[™] Analyser User Guide, 2010
6. W. R. Rodriguez, N. Christodoulides, P. N. Floriano, S. Graham, S. Mohanty, M. Dixon, M. Hsiang, T. Peter, S. Zavahir, I. Thior, D. Romanovicz, B. Bernard, A. P. Goodey, B. D. Walker and J. T. McDevitt, *PLoS Med.*, 2005, 2, e182.
7. M. Hosokawa, M. Asami, S. Nakamura, T. Yoshino, N. Tsujimura, M. Takahashi, S. Nakasono, T. Tanaka and T. Matsunaga, *Biotechnol. Bioeng.*, 2012, 109, 2017-2024.
8. X. Cheng, D. Irimia, M. Dixon, K. Sekine, U. Demirci, L. Zamir, R. G. Tompkins, W. Rodriguez and M. Toner, *Lab Chip*, 2007, 7, 170-178.
9. H. Zhu, M. Macal, C. N. Jones, M. D. George, S. Dandekar and A. Revzin, *Anal. Chim. Acta*, 2008, 608, 186-196.
10. U. A. Gurkan, T. Anand, H. Tas, D. Elkan, A. Akay, H. O. Keles and U. Demirci, *Lab Chip*, 2011, 11, 3979-3989.
11. D. Gao, H. F. Li, G. S. Guo and J. M. Lin, *Talanta*, 2010, 82, 528-533.
12. BD Bioscience, <https://www.bdbiosciences.com/documents/FACSPresto-BR.pdf>
13. ChipCare, http://www.globe-network.org/en/system/files/en/network/resource/8_fraser_chipcare_presentation.pdf
14. M. Murtagh, 2012 Hiv/Aids Diagnostic Technology Landscape: 2nd Edition, 2012
15. X. Cheng, D. Irimia, M. Dixon, J. C. Ziperstein, U. Demirci, L. Zamir, R. G. Tompkins, M. Toner and W. R. Rodriguez, *J. Acquir. Immune Defic. Syndr.*, 2007, 45, 257-261.
16. D. S. Boyle, K. R. Hawkins, M. S. Steele, M. Singhal and X. Cheng, *Trends Biotechnol.*, 2012, 30, 45-54.
17. M. Murtagh, 2012 Hiv/Aids Diagnostic Technology Landscape: Semi-Annual Update, 2012
18. World Health Organization, Who Expert Meeting on Short, Medium and Longer Term Product Development Priorities in Hiv-Related Diagnostics, 2012
19. D. Y. Stevens, C. R. Petri, J. L. Osborn, P. Spicar-Mihalic, K. G. McKenzie and P. Yager, *Lab Chip*, 2008, 8, 2038-2045.
20. E. Fu, B. Lutz, P. Kauffman and P. Yager, *Lab Chip*, 2010, 10, 918-920.
21. M. Hitzbleck, L. Gervais and E. Delamarche, *Lab Chip*, 2011, 11, 2680-2685.
22. L. Gervais and E. Delamarche, *Lab Chip*, 2009, 9, 3330-3337.
23. F. Brandl, F. Kastner, R. M. Gschwind, T. Blunk, J. Tessmar and A. Gopferich, *J. Control. Release*, 2010, 142, 221-228.
24. S. Ganta, H. Devalapally, A. Shahiwala and M. Amiji, *J. Control. Release*, 2008, 126, 187-204.
25. D. Puchberger-Enengl, C. Krutzler, F. Keplinger and M. J. Vellekoop, *Lab Chip*, 2014, 14, 378-383.
26. M. Tijero, R. Diez-Ahedo, F. Benito-Lopez, L. Basabe-Desmonts, V. Castro-Lopez and A. Valero, *Biomicrofluidics*, 2015, 9, 044124.
27. E. Jang, S. Kim and W. G. Koh, *Biosens. Bioelectron.*, 2012, 31, 529-536.

28. Z. Zhao, M. A. Al-Ameen, K. Duan, G. Ghosh and J. F. Lo, *Biosens. Bioelectron.*, 2015, 74, 305-312.
29. V. N. Luk, L. K. Fiddes, V. M. Luk, E. Kumacheva and A. R. Wheeler, *Proteomics*, 2012, 12, 1310-1318.
30. M. Mortato, L. Blasi, G. Barbarella, S. Argenti and G. Gigli, *Biomicrofluidics*, 2012, 6, 044107.
31. S. Cosson and M. P. Lutolf, *Sci. Rep.*, 2014, 4, 4462.
32. M. Beck, S. Brockhuis, N. van der Velde, C. Breukers, J. Greve and L. W. M. M. Terstappen, *Lab Chip*, 2012, 12, 167-173.
33. Microspheres Online, <http://microspheres.us/>
34. M. Hitzbleck and E. Delamarche, *Chem. Soc. Rev.*, 2013, 42, 8494-8516.
35. M. Singh, H. M. Haverinen, P. Dhagat and G. E. Jabbour, *Advanced Materials*, 2010, 22, 673-685.
36. E. Tekin, P. J. Smith and U. S. Schubert, *Soft Matter*, 2008, 4, 703-713.
37. B. J. de Gans, P. C. Duineveld and U. S. Schubert, *Advanced Materials*, 2004, 16, 203-213.
38. M. Beck, S. Brockhuis, N. van der Velde, C. Breukers, J. Greve and L. W. Terstappen, *Lab Chip*, 2012, 12, 167-173.
39. BioFluidix GmbH, Manual Pipejet Nanodispenser Kit, version 1.11
40. W. Streule, T. Lindemann, G. Birkle, R. Zengerle and P. Koltay, *J. Lab. Autom.*, 2004, 9, 300-306.
41. È. S. Benet, Msc Thesis: Printing of Microfluidic Chambers for Blood Cell Counting, University of Twente, 2012
42. X. Zhang, D. Wasserberg, C. Breukers, L. W. Terstappen and M. Beck, *Analyst*, 2016, 141, 3068-3076.
43. X. Zhang, D. Wasserberg, C. Breukers, L. W. Terstappen and M. Beck, *ACS Appl Mater Interfaces*, 2016,
44. T. Higuchi, *J. Pharm. Sci.*, 1961, 50, 874-&.
45. J. Siepmann, F. Lecomte and R. Bodmeier, *Journal of Controlled Release*, 1999, 60, 379-389.
46. J. Siepmann, F. Lecomte and J. Rathbone, *Fundamentals and Applications of Controlled Release Drug Delivery*, Springer, New York, 2012
47. P. V. Kozlov and G. I. Burdygina, *Polymer*, 1983, 24, 651-666.
48. F. Badii, C. Martinet, J. R. Mitchell and I. A. Farhat, *Food Hydrocolloids*, 2006, 20, 879-884.
49. M. Djabourov and P. Papon, *Polymer*, 1983, 24, 537-542.
50. F. Badii, W. MacNaughtan and I. A. Farhat, *Int. J. Biol. Macromol.*, 2005, 36, 263-269.
51. I. Yakimets, N. Wellner, A. C. Smith, R. H. Wilson, I. Farhat and J. Mitchell, *Polymer*, 2005, 46, 12577-12585.
52. P. Godard, J. J. Biebuyck, M. Daumerie, H. Naveau and J. P. Mercier, *J. Polym. Sci., Part B: Polym. Phys.*, 1978, 16, 1817-1828.
53. World Health Organization, Consolidated Guidelines on the Use of Antiretroviral Drugs for Treating and Preventing Hiv Infection, 2013

Chapter 6

Screening of polysaccharide coated microdevices for on-chip immunostaining

Xichen Zhang, Dorothee Wasserberg, Christian Breukers, Leon W. M. M. Terstappen, Markus Beck

Abstract

As shown in the previous chapters, incorporation of a gelatin matrix based reagent delivery system in a cell counting chamber can be used for a simple on-chip sample preparation for point-of-care CD4 counting. However, the conformational maturation of gelatin leads to continuous change in release kinetics of embedded antibody and unfavorable disruption in staining homogeneity, resulting in a short shelf life of the counting chambers. Here we explore the feasibility of replacing gelatin by a polysaccharide as an alternative matrix. Eleven matrix candidates including pure polysaccharides (chitosan, gellan, xanthan and carrageenan), polysaccharide blends (alginate/chitosan, pectin/chitosan, carrageenan/chitosan, gellan/chitosan and xanthan/chitosan) and ionically crosslinked polysaccharides (alginate/Ca²⁺ and pectin/Ca²⁺) were prepared and evaluated with regard to the following aspects: surface roughness, matrix integrity, antibody distribution and on-chip immunostaining. From these, gellan was selected as the most promising candidate. A rapid and homogeneous staining was successfully demonstrated in gellan coated counting chambers. For the assessment of shelf life, the counting chambers were stored at a temperature span from 4 to 40°C up to 12 weeks. Differences in antibody release from both fresh and aged gellan matrices stored under all different temperatures were found to be negligible, suggesting the excellent thermal stability of gellan. Gellan coated counting chambers show longer shelf lives with more reproducible immunostaining than comparable gelatin coated chambers.

6.1 Introduction

Microfluidics is an enabling technology for the development of *in vitro* point-of-care (POC) diagnostic tests using disposable microdevices.¹⁻³ Sample preparation via the integration and release of dry reagents in microdevices greatly reduces the dependency on the assistance of peripheral instruments and manual operation by skilled personnel. A variety of solutions have been developed to integrate dry reagents in microdevices and obtain on-chip sample-reagent mixing after the introduction of sample fluid.⁴ Conventional approaches focus on the defined patterning of dry reagents in sophisticatedly fabricated microdevices with well designed microchannels.^{5,6} They attain controlled reagent dissolution by directing sample fluid in complex flow paths. The cost of fabricating such delicate microdevices and including external flow control systems make those approaches incompatible with POC applications in low-resource settings. Paper based microfluidics is alternative and simple platforms^{7,8} to accommodate dry reagents and achieve desired dissolution which is enabled by capillary driven sample fluid. However, the flow path in the cellulose matrix has a high risk of being blocked in the presence of the sample fluid containing particles/cells with heterogeneous size. To overcome those limitations, simple microdevices already containing a hydrogel matrix with embedded reagents have been introduced to enable controlled reagent release upon the contact with sample fluid.⁹⁻¹³ For the enumeration of CD4 positive (CD4⁺) T-lymphocytes in a stopped flow configuration, we have previously reported cell counting chambers containing antibody embedded in a gelatin matrix.⁹ In this application, on-chip sample preparation is realized by flowing whole blood into a gelatin coated counting chamber by capillary force. Fluorophore-labeled antibody is released from the gelatin matrix after the completion of sample inflow and stains the cells of interest. The resulting controlled delay in release prevents antibody wash-off during sample inflow and released antibody is distributed homogeneously across the counting chamber. This is a prerequisite to ensure accurate cell count in our image cytometry concept. For analysis, a fluorescence imaging setup, with a wide field of view at low magnification, was used to capture a large sample volume in a single image. Therefore, stained cells can only be identified via their fluorescence intensity, not via their morphology. This

means that, if the staining is inhomogeneous, the definition of selection criteria, e.g. intensity threshold, becomes problematic.

Controlling and optimizing delayed release in a gelatin coated chamber that is expected to provide consistent results within a shelf life of many months is being complicated by the fact that gelatin undergoes continuous maturation (continuous formation of physical crosslinks). The formation of physical crosslinks is largely dependent on preparation and storage conditions (e.g. pressure, temperature and humidity).^{14,15} This ongoing maturation alters the structure of the gelatin matrix, in terms of mesh size. The material-dependent ageing of gelatin directly influences the antibody release over time and leads to inconsistent immunostaining, thus reducing the reproducibility and accuracy of the cell count.¹⁵

Polysaccharides are widely used in drug delivery studies due to their good biocompatibility, high abundance and low processing cost.^{16,17} In contrast to gelatin, no significant conformational change was found in polysaccharide matrices which were stored under dry and cool environments¹⁸, suggesting polysaccharides are stable with negligible ageing issues. In this paper, we screened polysaccharides to replace gelatin for use as release matrices in the counting chambers. Eleven polysaccharide candidates were divided into 3 groups: pure polysaccharides including chitosan (chi), gellan (gell), xanthan (xan) and carrageenan (carr); polysaccharide blends including alginate/chitosan (alg/chi), pectin/chitosan (pec/chi), carrageenan/chitosan (carr/chi), gellan/chitosan (gell/chi) and xanthan/chitosan (xan/chi); ionically crosslinked polysaccharides including alginate/ Ca^{2+} (alg/ Ca^{2+}) and pectin/ Ca^{2+} (pec/ Ca^{2+}). The materials were evaluated with regard to their surface roughness, matrix integrity, antibody distribution and on-chip immunostaining capability in comparison with the reference material, gelatin. Gellan was found to be the most promising matrix material and further tested for stability under different storage conditions. The present study shows that gellan meets all the criteria: low surface roughness, uniform antibody distribution before and after sample inflow, complete and homogeneous immunostaining and good thermal stability to enable reproducible CD4 counting in low-resource settings.

6.2 Experiments

6.2.1 Materials

All the chemicals were purchased from Sigma-Aldrich. Chitosan was dissolved in 0.4% v/v acetic acid at room temperature (RT, ~20°C) while stirring for 1 h. The other polysaccharide materials were dissolved in milliQ water at 80°C by while stirring for 1 h. Gelatin (type A, Bloom 295 g) was dissolved in milliQ water at 40°C while stirring for 1 h. All the solutions were left to cool to RT and then filtered gravitationally using a membrane filter (CellTrics®, mesh size 20 µm, Partec) before use. The resulting solutions of 0.1% w/v pure polysaccharides were ready to use. Next, polysaccharide blends were prepared by suspending polycationic chitosan with one of five polyanionic polysaccharides (alginate, pectin, carrageenan, gellan, xanthan) in milliQ water. The blends were equimolar mixtures of amine groups (-NH₂) in chitosan and carboxylic acid groups (-COOH) in its respective counterpart. In the case of suspensions of ionically crosslinked polysaccharides, CaCl₂ was added to alginate and pectin in milliQ water. The molar concentration of Ca²⁺ was half of the carboxylic acid groups (-COOH) in alginate and pectin in order to achieve the highest degree of crosslinking, aiming for the so-called egg-box conformation.^{19, 20} The total concentration of polysaccharides in blends and ionically crosslinked suspensions was only 0.02% w/v to prevent precipitation during mixing. Casting solutions were prepared by adding the supernatant of allophycocyanin labeled antiCD3 IgG (APC-αCD3, clone SK7, 260 kDa, BD) and peridinin chlorophyll labeled antiCD4 IgG (PerCP-αCD4, clone MEM241, 308kDa, Exbio) solutions after centrifuge (21000×g for 3 min) to each of the above polysaccharide solutions.

6.2.2 Counting chamber fabrication and matrix preparation

A solution of each polysaccharide was cast and dried to form a layer of matrix with ~50 nm thickness in the central region. As illustrated in Fig. 6.1, precut laminating adhesive (nominal thickness 25.4 µm, 3M) was attached to a glass substrate (Menzel, 76 mm × 26 mm × 1 mm) to create a confined casting area (15 mm × 9 mm). Into this area, 20 µL of 0.15% gelatin, 24 µL of 0.1% pure polysaccharide or 120 µL of 0.02% polysaccharide blend and ionically crosslinked polysaccharide was cast and dried

under ambient conditions. All cast matrices contained 60 ng of APC- α CD3 and 12 ng of PerCP- α CD4. Following casting, a capping glass slide (Menzel, 76 mm $\times$ 11 mm $\times$ 1 mm) was attached as a cover to create a complete counting chamber.

Surface roughness, matrix dissolution, antibody distribution in the matrices before sample inflow and the fraction of released antibody were determined on uncovered chambers, while the determination of the profile of fluorescence intensity after sample inflow and the characterization of on-chip immunostaining were performed on complete chambers.

Fig. 6.1: Schematic representations of cell counting chambers: a) Perspective view, b) Side view.

6.2.3 Determination of surface roughness

To quantify the surface roughness of the matrices, the coefficient of variation (CV) of the thickness was determined from a representative region with an area size of 2 mm². The matrix thickness was measured using a white light interferometer (smartWLI microscope, GBS). Scraping off a small area of the matrix in the central region allowed us to use the substrate as a reference. Fig. 6.2 shows the topography of three representative matrices of pure polysaccharide, polysaccharide blend and ionically crosslinked polysaccharide.

Fig. 6.2: Topography of three representative matrix materials with characteristic surface profiles, a) chitosan, b) carrageenan/chitosan and c) alginate/ Ca^{2+} .

6.2.4 Fluorescence imaging

Fluorescence images of the matrices were taken using a custom-built fluorescence imaging system.⁹ The image area is $7.8 \text{ mm} \times 5.2 \text{ mm}$ with a resolution of $10 \text{ }\mu\text{m}$. The fluorescence intensity of APC- αCD3 at each pixel from a representative region ($4.1 \text{ mm} \times 3.1 \text{ mm}$) was recorded after background subtraction. The intensity histograms obtained from the defined region were normalized to the mean intensity of the region. The resulting CV of the intensity is indicative of the homogeneity of the APC- αCD3 distribution in the matrix.

6.2.5 Determination of matrix dissolution during sample inflow

To quantify matrix dissolution during sample inflow, the matrix was immersed in phosphate buffered saline (PBS), which proved to be a suitable replacement of blood plasma in the release study¹⁴, for 10 s (the estimated time for sample inflow to complete in the counting chambers) while stirring. The average thickness of a defined region (2 mm^2) before dipping was measured. After dipping and a subsequent gentle blow-drying step, the average thickness of the same region was measured again and the fractions of retained matrix material after dipping were obtained.

6.2.6 Measurement of fluorescence profiles after sample inflow and on-chip immunostaining

To quantify wash-off of antibody during sample inflow, fluorescence images before and after inflow were compared: First, a fluorescence image (APC- α CD3 readout) of each matrix material in the counting chamber before sample inflow was recorded. Then, approximately 5 μ L of finger prick blood was added into the counting chamber through the filling port by capillary forces. Blood samples were collected from healthy individuals at the University of Twente, all of whom had provided written informed consent in advance. All experiments were performed in compliance with the relevant laws and institutional guidelines. Immediately after sample inflow had ended, a second fluorescence image of the filled counting chamber was taken. As described in Chapter 3, normalized intensity profiles were obtained from the ratios of fluorescence intensities of the filled versus the unfilled chamber at each corresponding position along the inflow direction in the background corrected images. This normalized image is assumed to depict the ratio between antibody concentration after and before sample inflow. After 30 min incubation in the counting chamber, a third image (both APC- α CD3 and PerCP- α CD4 readout) was taken and image analysis software (using ImageJ)²¹ was used to identify CD4⁺ T-lymphocytes (APC- α CD3 and PerCP- α CD4 double positive objects).

6.2.7 Quantification of the fraction of released antibody during sample inflow and incubation

To quantify the fraction of embedded antibody that is released from each matrix, fluorescence images of different dry matrix materials with embedded APC- α CD3 were taken and the average intensities of defined matrix regions (4.1 mm $\times$ 3.1 mm) were determined. Then, the matrices were immersed in PBS while stirring for 10 s (inflow period). Following a gentle blow-drying step, the average intensities of the same matrix regions were determined again. A second round of additional 30 min immersion was carried out for all matrices. Likewise, the average intensities of the same matrix regions were determined. The intensities of each matrix after 10 s and 30 min PBS immersion were compared with the intensity of the dry matrix. The

determined intensity ratio of the matrix before and after immersion is indicative of the fractional release of APC- α CD3 after 10 s ($M_{10\text{ s}}/M_{\text{total}}$) and after 30 min ($M_{30\text{ min}}/M_{\text{total}}$).

6.2.8 Determination of shelf life of matrix coated counting chambers

To determine the shelf life of the counting chambers, the consistency of antibody release kinetics from the matrix was tested first. The release fractions of APC- α CD3 after 10 s and 30 min from both fresh and aged matrices (up to 12 weeks) stored with silica gel (10% relative humidity) in dark and airtight containers at 4°C, 20°C and 40°C were measured as described above. The development in antibody release over storage time gives an indication of the functional stability of the matrix. Next, the stability of embedded antibody and the reproducibility of immunostaining were evaluated. Average fluorescence intensities (both APC- α CD3 and PerCP- α CD4 readout) of fresh and aged matrices with embedded antibodies before sample inflow were determined. After sample inflow, average fluorescence intensities of CD3⁺ (red excitation) and CD4⁺ (blue excitation) T-lymphocytes in blood samples were obtained from images taken after 30 min incubation at RT. The fitting of intensity decay is indicative of the shelf life of the counting chambers.

6.3 Results and discussion

Criteria for complete on-chip sample preparation in our cell counting chambers are: precise on-chip sample metering, sufficient sample volume ($> 1\text{ }\mu\text{L}$) in one image and homogeneous distribution of antibody. Sample metering can be realized by the design of the chamber frame and bubble free sample filling. Since the height of the counting chamber is only $\sim 27\text{ }\mu\text{m}$. A large imaging area ($> 37\text{ mm}^2$) is therefore required for a sufficient sample volume per image. Using affordable optical components, such as a wide field of view, only allows for low resolution imaging, restricting the identification of stained cells to their fluorescence intensities only. In this case, uniform intensities of stained cells as well as an, at least locally, homogeneous background intensity, i.e. homogeneous antibody distribution, are essential.

6.3.1 Characterization of dry polysaccharide matrices with embedded antibody

To prevent the formation of air bubbles during sample inflow, the surface of the matrix should be smooth to avoid spatial variation of inflow speed. The roughness of each matrix material is presented in Table 6.1. Well dissolved chitosan, gellan and carrageenan solutions can be obtained with mild pH adjustment or heat treatment. The resulting matrices show smooth surfaces comparable with the reference material, gelatin, and are believed to facilitate sample filling. Poorly dissolved xanthan, polysaccharide blends and ionically crosslinked polysaccharides formed suspensions of aggregates and resulted in great surface roughness, which prevents homogeneous filling of the counting chambers.

Table 6.1: matrix roughness (CV in thickness)

gelatin	chi	gell	xan	carr	alg/chi
0.051	0.034	0.066	0.203	0.107	0.228
pec/chi	carr/chi	gell/chi	xan/chi	alg/Ca ²⁺	pec/Ca ²⁺
0.336	0.368	0.294	0.351	0.146	0.167

The antibody distribution in the dry matrices is shown in Fig. 6.3. A narrow distribution of fluorescence intensities ($CV \leq 0.11$) is found in gelatin, chitosan, gellan and carrageenan, suggesting uniform distribution of antibody in the matrices, while an inhomogeneous distribution of antibody is found in xanthan, polysaccharide blend and ionically crosslinked polysaccharide matrices. Fig. 6.4 shows the relation between the CV in thickness and the CV in fluorescence intensity. The good correlation between the two CVs indicates that there probably is no significant phase separation between antibody and polysaccharide in the matrix.

Fig. 6.3: Histograms of fluorescence intensity of APC-αCD3 in polysaccharide matrices.

Fig. 6.4: Correlation of CV in roughness and CV in fluorescence intensity of APC-αCD3 in polysaccharide matrices.

The roughness and antibody distribution in the dry matrices of chitosan, gellan and carrageenan were deemed superior to those of the other candidates and were equally good as the corresponding values in gelatin. These three materials were therefore selected for further evaluation (see below).

6.3.2 Characterization of antibody distribution in the counting chambers after sample inflow

During sample inflow, antibody could be released from the matrix and washed toward the venting port of the counting chamber. As a result, an antibody concentration gradient along the inflow direction in the counting chamber is generated and will have an adverse impact on the homogeneity of immunostaining. The preservation of homogenous antibody distribution in the counting chamber after sample inflow is essential and can be evaluated by quantifying the antibody wash-off during sample inflow. In Fig. 6.5a, the measured ratio of antibody concentrations before and after sample inflow is plotted against the distance from the filling port of the counting chamber. A dramatic increase in antibody concentration along the direction of sample inflow is found in the counting chambers coated with carrageenan, indicating a severe inhomogeneity of the distribution. The wash-off of antibody from gellan results in less inhomogeneity, slightly better than the reference, gelatin. In the chitosan matrix, antibody wash-off is largely prevented.

The reason for the strong antibody wash-off in the case of carrageenan is probably due to the dissolution of the matrix. As shown in Fig. 6.5b, more than 60% of the matrix dissolved in PBS during the inflow period (~ 10 s), leading to a substantial fraction of antibody being released. The APC- α CD3 was readily swept from the “filling region” (0-1 mm from the filling port) toward the “venting region” (0-1 mm from the venting port) by the sample inflow, thus resulting in a steep concentration gradient (~ 33 -fold increase as indicated by the red curve in Fig. 6.5a). With respect to the dissolution resistant matrices, both gellan and chitosan outperformed gelatin as matrix materials in retaining antibody during sample inflow and led to more gradual concentration gradients than gelatin. Chitosan was the most successful matrix in delaying the release of antibody to preserve a homogeneous distribution after inflow.

Based on the evaluation of the above characteristics, carrageenan is not suitable

as matrix material and chitosan and gellan were selected for further screening.

Fig. 6.5 a) Normalized and background corrected intensity ratios indicating the distribution of APC- α CD3 in the counting chambers coated with different matrices after/before sample inflow. b) Ratios of dissolved matrix material and fractional antibody release after sample inflow. Data points represent mean $\pm$ standard deviation ($n = 3$).

6.3.3 Evaluation of on-chip immunostaining

To obtain a test result within a reasonable time in the POC diagnostic tests, on-chip immunostaining is required to approach saturation within 30 min. Therefore staining efficiency was assessed by quantifying the intensity of stained cells in chitosan and gellan coated counting chambers after 30 min incubation. The scatter plots of the fluorescence intensities of cells stained with APC- α CD3 (red excitation) vs. PerCP- α CD4 (blue excitation) were analyzed. Fig. 6.6 shows that double positive cells form a clearly defined population in exemplary gellan and gelatin coated counting chambers. Intensities of stained cells in gellan coated chambers are close to those in gelatin coated chambers (intensities of cells in gellan chambers over those in gelatin chambers: $93 \pm 6\%$ in APC readout, $79 \pm 7\%$ in PerCP readout, $n = 3$). In contrast, the stained cells in chitosan coated counting chambers were much dimmer (intensities of cells in chitosan chambers over those in gelatin chambers: $13 \pm 6\%$ in APC readout, $22 \pm 3\%$ in PerCP readout, $n = 3$), indicating incomplete staining.

The resulting staining intensities for each of the three materials agree with the fractional release data after 30 min immersion ($M_{30 \text{ min}}/M_{total}$). The majority of antibody was released from the gelatin ($94 \pm 5\%$, $n = 3$) and gellan ($95 \pm 2\%$, $n = 3$) matrices and became available to stain cells within the 30 min incubation time. Yet,

only a small portion ($24 \pm 3\%$, $n = 3$) of antibody was released from the chitosan matrix after 30 min. Clearly, the much longer delay in antibody release in chitosan, compared to gellan and gelatin reduces the amount of available antibody to such an extent that saturation could not be achieved within the given incubation period. Additionally, the substantial amount of antibody retained in the chitosan matrix contributed to the background intensity. The increased background intensity together with the reduced cell intensity resulted in a decreased cell to background intensity ratio, which made the identification of stained cells in chitosan coated chambers more difficult. For this reason, gellan was selected as the most promising candidate to replace gelatin as the matrix for the storage and delayed release of antibody in the counting chambers.

Fig. 6.6: Scatter plots and histograms of fluorescence intensity of APC- α CD3 (red excitation) and PerCP- α CD4 (blue excitation) stained cells obtained from the counting chambers coated with: gelatin (left), gellan (center) and chitosan (right) after 30 min incubation. The purple circles are those cells that were identified as the double positive cell population.

6.3.4 Thermal stability

In order to evaluate the suitability of gellan for use in POC diagnostic tests, the shelf life of the material needed to be assessed. To this end, the influence of material ageing on the kinetics of antibody release from gellan matrix was tested. The fractional release of APC- α CD3 from gellan stored at three different temperatures was measured for different periods of storage time up to 84 days. As in the above experiments, the two characteristic release fractions after 10 s (inflow period) and 30 min (incubation period) were recorded. Fig. 6.7 shows that $M_{10\text{ s}}/M_{\text{total}}$ and $M_{30\text{ min}}/M_{\text{total}}$ are storage

temperature and time independent, indicating the excellent stability of gellan as matrix material. Subjected to given storage condition (10% RH, temperature from 4 to 40°C), aged gellan was as reliable as the fresh matrix to release antibody for on-chip immunostaining, as neither the fraction of released antibody during the first 10 s (inflow period) nor the fraction during 30 min (incubation period) changes significantly within 84 days of storage.

Fig. 6.7: Fractional release of APC-αCD3 after 10 s ($M_{10\text{ s}}/M_{\text{total}}$, open circles) and after 30 min ($M_{30\text{ min}}/M_{\text{total}}$, solid circles) incubation from gellan matrices stored under 10% RH conditions at 4°C (blue symbols), 20°C (green symbols) and 40°C (red symbols). Data points represent mean $\pm$ standard deviation ($n = 3$).

Storage time-dependent cell and background intensities obtained from gellan coated counting chambers subject to different temperatures are summarized in Fig. 6.8. The parallel progressions of the decay of cell intensity and background intensity suggest that the degradation of the fluorophore governs the reduction of maximally attainable average cell staining intensity. By quantifying the decay rate, we noticed a temperature-dependent effect on the cell staining. At 40°C, the degradation of fluorophore-labeled antibody is the fastest (half life: 50-100 days). At 4°C, a half life of over 250 days can be reached. The half life at 20°C (150-250 days) lies in between. Within this half life period, i.e. shelf life, stained cells can still be consistently detected in spite of reduced intensities.

Fig. 6.8: Average fluorescence intensities of cells stained with APC- α CD3 (red circles) and PerCP- α CD4 (blue circles) in gellan/antibody coated counting chambers stored at room 4°C (left), 20° (center) and 40°C (right). The number of counted cells is represented by the size of the bubbles. Solid lines are fitting curves of the decay of cell intensities. Average background intensities of dry gellan matrices (red excitation: pink squares, blue excitation: cyan squares). Data points represent mean $\pm$ standard deviation ($n = 3$). Dashed lines are fitting curves of the decay of background intensities.

6.4 Conclusion

We set out to identify alternatives for gelatin as a matrix material for CD4 counting chambers. Eleven different polysaccharides have been tested as potential matrix materials. They were divided into three groups: pure polysaccharides, polysaccharide blends and ionically crosslinked polysaccharides. Among these candidates, only gellan meets all criteria required for a matrix material to achieve complete and homogeneous immunostaining within 30 min in the counting chambers. Compared with gelatin, gellan shows comparable surface roughness and matrix integrity, but outperforms gelatin in terms of homogeneity of antibody distribution and long term stability.

References

1. V. Gubala, L. F. Harris, A. J. Ricco, M. X. Tan and D. E. Williams, *Anal. Chem.*, 2012, 84, 487-515.
2. C. D. Chin, V. Linder and S. K. Sia, *Lab Chip*, 2012, 12, 2118-2134.
3. B. Weigl, G. Domingo, P. Labarre and J. Gerlach, *Lab Chip*, 2008, 8, 1999-2014.
4. M. Hitzbleck and E. Delamarche, *Chem. Soc. Rev.*, 2013, 42, 8494-8516.
5. M. Hitzbleck, L. Gervais and E. Delamarche, *Lab Chip*, 2011, 11, 2680-2685.
6. E. Garcia, J. R. Kirkham, A. V. Hatch, K. R. Hawkins and P. Yager, *Lab Chip*, 2004, 4, 78-82.
7. D. Y. Stevens, C. R. Petri, J. L. Osborn, P. Spicar-Mihalic, K. G. McKenzie and P. Yager, *Lab Chip*, 2008, 8, 2038-2045.
8. E. Fu, B. Lutz, P. Kauffman and P. Yager, *Lab Chip*, 2010, 10, 918-920.
9. M. Beck, S. Brockhuis, N. van der Velde, C. Breukers, J. Greve and L. W. M. M. Terstappen, *Lab Chip*, 2012, 12, 167-173.
10. M. Mortato, L. Blasi, G. Barbarella, S. Argentiore and G. Gigli, *Biomicrofluidics*, 2012, 6, 044107.
11. M. Tijero, R. Diez-Ahedo, F. Benito-Lopez, L. Basabe-Desmonts, V. Castro-Lopez and A. Valero, *Biomicrofluidics*, 2015, 9, 044124.
12. H. T. Mitchell, S. A. Schultz, P. J. Costanzo and A. W. Martinez, *Chromatography*, 2015, 2, 436-451.
13. R. R. Niedl and C. Beta, *Lab Chip*, 2015, 15, 2452-2459.
14. X. Zhang, D. Wasserberg, C. Breukers, L. W. M. M. Terstappen and M. Beck, *Analyst*, 2016, 141, 3068-3076.
15. X. Zhang, D. Wasserberg, C. Breukers, L. W. Terstappen and M. Beck, *ACS Appl. Mater. Interfaces*, 2016, 8, 27539-27545.
16. E. Chornet and S. Dumitriu, *Adv. Drug Deliv. Rev.*, 1998, 31, 223-246.
17. T. Coviello, P. Matricardi, C. Marianecci and F. Alhaique, *J. Control. Release*, 2007, 119, 5-24.
18. G. Franz and U. Feuerstein, *Macromol. Symp.*, 1997, 120, 169-181.
19. I. Braccini and S. Perez, *Biomacromolecules*, 2001, 2, 1089-1096.
20. C. K. Kuo and P. X. Ma, *Biomaterials*, 2001, 22, 511-521.
21. C. A. Schneider, W. S. Rasband and K. W. Eliceiri, *Nat. Methods*, 2012, 9, 671-675.

Chapter 7

Fully printed microfluidic devices enabling on-chip reagent storage and sample preparation for simple CD4 counting

Xichen Zhang, Dorothee Wasserberg, Christian Breukers, Leon W. M. M. Terstappen, Markus Beck

Abstract

Hydrogel matrices have shown to bear excellent properties for stable preservation and tunable release of biological reagents. Therefore, the integration of hydrogel matrices into microfluidic devices can substantially simplify the conventional sample preparation in miniaturized bioassays. In this paper, we present a gellan/trehalose coated microfluidic chamber with on-chip sample preparation for point-of-care (POC) CD4 counting. The counting chambers are produced in an automatic manner. Chamber frames are created by printing a suspension of microbeads acting as spacers in UV curable glue on a substrate glass slide. A gellan/trehalose solution containing antibodies is deposited within the chamber frame using inkjet printing and forms a matrix after drying. The chambers are assembled by adding a capping glass slide on the printed frames and subsequent curing with UV light using a robotic assembly setup. The chamber design allows for simple sample filling by capillary action. The analyzed sample volume is defined solely by the image area and chamber height, making the assay independent of precision sample handling and external equipment. The matrix stabilizes the fluorophore of the labeled antibody during storage and enables delayed antibody release for optimal on-chip cell staining. After 30 min incubation, fluorescence images of the stained cells are recorded using a custom-built image cytometer. The obtained cell counts of CD4 positive (CD4⁺) T-lymphocytes show excellent agreement with the results from flow cytometry, the gold standard.

7.1 Introduction

On-chip sample preparation performed in a microfluidic device independent of skilled laboratory operators and external equipment shows a great potential to reduce the complexity of benchtop biological assays, thus making this concept of sample preparation very well suited for POC diagnostics, i.e. immunostaining and biomolecule sensing. In the most simplistic approach, biological reagents are directly deposited and dried in a microfluidic device. Subsequently, sample fluid is filled into the device by capillary force to dissolve the reagents and the biochemical reaction is initiated in the sample solution. The result is that peripheral pumping systems for sample loading and reagent supply, which are used in conventional microfluidic devices, become obsolete. In this sample preparation concept, controlled release of reagents into the sample solution is critical for the optimal reaction inside a microfluidic device with a stopped flow configuration. However, the defined dissolution of reagents into the sample solution is challenging in microfluidic devices without fluid actuation. Advanced geometry of flow paths have been designed in paper-based microfluidic devices¹ and hydrophilic microchannel networks² to improve dissolution controlled reagent release. However, fragile biological reagents, e.g. enzymes and antibodies, directly deposited in the microfluidic devices often endure major degradation upon interaction with the bare substrate. Extensive studies in drug delivery have demonstrated that both naturally and synthetically derived hydrogels are capable of storing and releasing a variety of reagents (e.g. drugs, DNA and proteins) without impairing their functionality.³⁻⁶ Due to their excellent biocompatibility, those hydrogel materials are suitable matrices to preserve biological reagents in self-contained microfluidic devices for long-term storage.^{7,8} In addition, they can also enable on-chip diffusion controlled reagent release which is initialized by matrix swelling and the resulting change of pore size due to rehydration or pH shift.⁹⁻¹¹ Recently, integration of hydrogel matrices to embed and release biological reagents in microfluidic devices has been shown to achieve a multitude of simple and rapid biosensing tests.¹²⁻¹⁵

A variety of methods have been developed to integrate hydrogel matrices and biological reagents in microfluidic devices.¹⁶ Among them, inkjet printing, a good alternative to lithographic techniques for patterning, has been well established for

hydrogel deposition^{17, 18} and extensively used in patterning biological reagents for bioassays¹⁹⁻²¹. Inkjet printing can deposit small amounts of ink with precise lateral resolution (10-100 μm).²² It can be used to dispense a large and diverse range of materials, and it is able to perform batch production in a cost-effective manner. Therefore, it is a powerful and versatile tool to achieve the integration of reagent-embedded hydrogel matrices in microfluidic devices.

Here, we developed a fully printed microfluidic device and demonstrated its performance with a simple CD4 counting assay based on on-chip immunostaining and image cytometry. The frame of the chamber was created by printing a suspension of polystyrene microbeads in UV curable glue to create chamber frames on a substrate glass slide and, subsequently, curing the suspension after the addition of a capping glass slide. The diameter of the microbeads defines the height of the chamber, which together with the image area, precisely defined by the optics of the fluorescence imager, contributes to a defined sample volume. Inside the chamber, a printed layer, made up of a composite gellan/trehalose matrix, was used to preserve the fluorophore of the labeled antibody. The way samples were prepared on-chip has been described previously.⁹ Briefly, finger prick blood is directly added to one end of the chamber and the blood fills the chamber driven by capillary forces. Upon contact with the blood sample, the matrix material will absorb water and start swelling. This rehydration enables the embedded antibody to diffuse through the matrix and be released into the blood to stain the cells of interest. We show here that optimizing on-chip immunostaining will eventually lead to a reliable cell counting assay, with cell counts comparable with those obtained from standard flow cytometry.

7.2 Materials and methods

7.2.1 Inkjet printing of antibody-embedded composite layers

To prepare the ink, gellan powder (Gelzan™ CM, Sigma) was dissolved in milliQ water at 80°C for 1 h while stirring and the obtained solution was filtered gravitationally using a membrane filter (CellTrics®, mesh size 20 μm , Partec) after cooling to room temperature (RT). Trehalose (Merck KGaA) was dissolved in milliQ water at 20°C by extensive vortexing. The stock solutions of allophycocyanin labeled antiCD3 IgG (APC-

α CD3, clone SK7, 260 kDa, BD) and peridinin-chlorophyll labeled antiCD4 IgG (PerCP- α CD4, clone MEM241, 308 kDa, Exbio) were centrifuged at 21000 $\times$ g for 3 min at 20°C and the supernatant was used. To determine the optimal mass ratio between trehalose and gellan in the composite matrix for antibody storage and delivery, varying amounts of trehalose were added to the ink solutions to reach mass fractions of trehalose in the matrix substance in the range from 0%, 9%, 17%, 33% to 50%. While the final concentrations of gellan, APC- α CD3 and PerCP- α CD4 in the ink solutions were 0.3% w/v, 0.42 μ g mL⁻¹ and 0.14 μ g mL⁻¹ respectively.

Inkjet printing was performed using an industrial inkjet printer (LP50, PiXDRO, Meyer Burger B.V.) with a Konica Minolta 512MHX printhead (nozzle diameter 27 μ m). A standard microscopy glass slide (76 mm $\times$ 26 mm $\times$ 1 mm, Menzel) was used as a substrate for ink deposition. Droplets of $\sim$ 20 pL ink solution were dispensed in 25 swaths of (360 dpi)² resolution each and a delay of 84 s between them, resulting in a final resolution of (1800 dpi)². These parameters, resulting in an average layer thickness in the range from $\sim$ 370 to $\sim$ 560 nm after drying (dispensed volume per area 0.1 μ L mm⁻²; gellan concentration 3 μ g μ L⁻¹, trehalose concentration 0 to 3 μ g μ L⁻¹, gellan density 0.8 g cm⁻³, trehalose density 1.6 g cm⁻³), were chosen to minimize puddle formation while maintaining sufficient throughput to keep nozzles jetting reliably.

7.2.2 Characterization of printed layers

Prior to the attachment of capping slides, the printed layer in each chamber was scratched and a representative region of interest (ROI) was selected along the scratch. The fluorescence image of each ROI on the printed layer was recorded in our custom-built fluorescence imager.⁹ The image area is 7.8 $\times$ 5.2 mm² (1530 $\times$ 1020 pixels) with a resolution of $\sim$ 10 μ m. The average fluorescence intensity of both APC- α CD3 and PerCP- α CD4 in the representative ROIs (800 $\times$ 600 pixels) were determined after background subtraction from the aforementioned fluorescence images. The obtained fluorescence intensities were used to evaluate the effect of trehalose on the preservation of the fluorophores (fluorescent proteins) in gellan layers.

The topography of the printed layer was determined using a white light interferometer (smartWLI-microscope, GBS). The average thicknesses of the same

representative ROIs were recorded. The topography and fluorescence image of a printed layer are shown in Fig. 7.1a.

Antibody wash-off and the integrity of the printed layer during sample inflow was tested as follows. The layer was dipped in phosphate buffered saline (PBS), a suitable replacement of blood plasma in the release study²³, with stirring for 10 s (inflow period). After a gentle blow-drying step, another fluorescence image of the layer was taken and the average fluorescence intensity of the same region after dipping was recorded. Likewise, the thickness of the layer at the same region was determined again. Comparing the parameters of the layer acquired before and after the dipping step yielded the amount of antibody wash-off and matrix dissolution.

7.2.3 Frame printing and counting chamber assembly

The chamber frames were printed around the areas containing printed layers on the glass substrates. The ink for frame printing was a suspension of acrylate-based UV curable glue (Byllux 6308, Byla GmbH) and 1% w/w monosized crosslinked polystyrene beads (diameter: 30 μm , Calibre® CS 30, Microbeads AS). A custom-built assembly and UV-curing setup including a piezo-driven dispenser (PipetteJet P9, BioFluidix GmbH) was employed to deposit the glue/bead suspension that constitutes the chamber frames. A more detailed description of the fabrication procedure can be found in Chapter 5. Briefly, droplets of ~ 8 nL were dispensed in a zigzag pattern with 0.5 mm drop-to-drop distance on the substrate. The process was optimized to produce intact lines resulting, in turn, in fully sealed chamber boundaries. The pattern in which the glue/bead suspension was dispensed is illustrated in Fig. 7.1c. The resulting frame encloses the printed hydrogel/antibody layer across the entire chamber region. Following frame printing, a capping glass slide (76 mm $\times$ 11 mm $\times$ 1 mm, Menzel) was attached to the substrate, creating filling and venting ports. The completed chamber was then exposed to UV light (wavelength: 350-400 nm) for 1 s through a 60 g glass weight, which was added for sufficient compression to ensure sealing. Chamber heights determined individually per chamber by laser interferometry (detailed description in Chapter 5) were found to be 30.7 ± 1.2 μm ($n = 67$).

Fig. 7.1: a) Left, Topography of a segment of a printed layer; Right, An overlay fluorescence image (red excitation (APC) and blue excitation (PerCP) of the same layer segment. Scale bars 0.1 mm. b) Schematic representation of a counting chamber. c) Left, Designed pattern to deposit the glue/bead suspension for the fabrication of chamber frames (black dots represent centers of drop positions, circles represent the estimated area covered by the droplet after being deposited on the glass substrate). Right, Bright field image of a segment of a cured frame.

7.2.4 Evaluation of on-chip immunostaining

Immunostaining experiments on whole blood samples were performed in counting chambers coated with different printed layers as described above. Finger prick blood was collected from anonymous healthy individuals at the University of Twente, all of whom had provided written informed consent in advance. All experiments were performed in compliance with the relevant laws and institutional guidelines. Blood samples were introduced into the chambers from the filling port and the filling completed within 10 s. Once the chamber was filled, ports were sealed with mineral oil to prevent drying. After 30 min incubation at RT, a set of fluorescence images of both APC and PerCP readouts were recorded. Automated image analysis using ImageJ²⁴ was executed to identify objects (2-9 pixels in diameter) with the fluorescence intensity of a least one of the readout signals exceeding the respective background level significantly. Each object (i.e. each identified cell) was quantified with regard to its integrated intensities for both fluorophores after background subtraction. Objects with above-threshold fluorescence intensities for both fluorophores were assigned to be CD4⁺ T-lymphocytes.

7.2.5 Comparison of CD4 counts acquired from image cytometry tests with flow cytometry analysis

To benchmark our approach against flow cytometry, blood tests were performed. For flow cytometry, blood samples were incubated with an antibody cocktail (CD3/CD4/CD8/CD45 Multitest™, BD) and a lysis buffer (FACS™ Lysing Solution, BD) in test tubes (Trucount™ Absolute Counting Tubes, BD) according to the specifications of the manufacturer. After incubation and lysis, samples were analyzed in the flow cytometer (FACS ARIA 2, BD). The measurement was stopped when 5000 beads were counted which corresponded to ~5 μ L sample volume.

In our image cytometry approach, the number of all CD4⁺ T-lymphocytes within the complete image area was counted. The known image area together with the known height of each chamber makes a volumetric (~1.2 μ L) quantification of the CD4 count possible. All CD4 counts are given as the number of CD4⁺ T-lymphocytes per μ L.

In order to compare the results from these two approaches in a large range of CD4 counts, in particular for low CD4 counts, which are clinically more relevant, blood samples with reduced leukocyte concentrations were prepared. First of all, whole blood was centrifuged at 1000×g for 15 min to separate blood plasma, leukocytes (buffy coat) and erythrocytes. After removing the buffy coat, the remaining plasma and erythrocyte fractions were remixed to yield leukocyte-depleted blood samples ("0%" leukocytes). By diluting the initial whole blood with leukocyte-depleted blood at different ratios, blood samples with 0%, 17%, 33%, 50%, 66%, 83% and 100% of the initial leukocyte concentration were obtained and tested using flow cytometry and our image cytometry approach.

7.3 Results and discussion

7.3.1 Fluorescence recovery of fluorophore-labeled antibody in printed layers

Trehalose, which is a non-reducing disaccharide, is commonly used to preserve biomolecules in the dry state, in particular for the stabilization of lyophilized proteins.²⁵⁻²⁷ Additionally, gellan as a biocompatible polysaccharide matrix has been reported to achieve controlled protein release in the past.^{28, 29} We chose to combine

the benefit of the capacity of trehalose for preserving proteins with the advantage of gellan to control protein release and use gellan/trehalose mixtures to produce composite layers to embed fluorophore-labeled antibody.

It is widely acknowledged that the function of proteins relies on the integrity of their structures, i.e. the preservation of intrinsic folds.³⁰ The conformation transition of the fluorescent proteins, i.e. protein unfolding, is associated with the change of their fluorescence intensity.^{31,32} Based on that fact, fluorescence quenching has been used to monitor the structural change of fluorescent proteins.^{33, 34} In this paper, the fluorescence intensity of the dry printed layer is used to assess the integrity of the fluorescent proteins (APC or PerCP). As can be clearly seen in Fig. 7.2, the addition of trehalose to a gellan matrix helps to preserve both APC and PerCP. Pure gellan quenches the fluorescence of APC and PerCP by a factor of ~ 3.5 and ~ 3 , respectively. Fluorescent proteins in layers containing 33% of trehalose or more seem to stay largely intact.

Fig. 7.2: Fluorescence intensities of dry printed layers with varying fractions of trehalose and the same amount of gellan and fluorophore-labeled antibodies. Data points represent mean $\pm$ standard deviation ($n = 4$).

7.3.2 Layer dissolution and antibody release during sample inflow

Trehalose is a highly water soluble molecule with a very small molecular weight of 342 Da.³⁵ It is readily dissolved upon the contact with blood samples and partially

disintegrates the matrix while dissolving.

To determine the layer dissolution and antibody release during sample inflow, printed layers were immersed in PBS for 10 s. The resulting fractional changes in thickness and fluorescence intensity of the printed layers are shown in Fig. 7.3. The more trehalose a printed layer contains, the more it dissolves upon the contact with PBS, resulting in more antibody release. This indicates that the presence of trehalose destabilizes the matrix and leads to substantial antibody wash-off during sample inflow, which results in inhomogeneous cell staining in the chamber.

Fig. 7.3: Dissolution of printed layers and the reduction of fluorescence intensities during sample inflow (10 s). Data points represent mean $\pm$ standard deviation ($n = 4$).

7.3.3 Optimization of on-chip immunostaining

The addition of trehalose to the gellan matrix shows improved protein preservation, while it increases antibody wash-off and therefore reduces the uniformity of cell staining throughout the chamber. To achieve intense and homogeneous on-chip immunostaining, the fraction of trehalose in the composite matrix has to be optimized. To this end, composite matrices with 17% and 33% trehalose together with a pure gellan matrix (negative control) were compared in blood tests. The evaluation of on-chip immunostaining was carried out by quantifying the intensity of stained cells in the counting chambers after 30 min incubation at RT with finger prick blood. The

fluorescence intensities of APC- α CD3 (red excitation) and PerCP- α CD4 (blue excitation) stained cells were analyzed and are shown in Fig. 7.4a. Cells stained in gellan/trehalose (17% and 33%) coated chambers are much brighter than pure gellan coated chambers, showing that the preservation of fluorophores (APC and PerCP) in printed layers contributes to the improved immunostaining. Fig. 7.4b and 7.4c show that double positive cells form defined and compact populations in pure gellan and gellan/trehalose (17%) coated chambers. However, a smeared-out double positive cell population (Fig. 7.4d) is observed in gellan/trehalose (33%) coated chambers. This large stretch in cell staining is indicative of antibody wash-off, implying severe staining inhomogeneity throughout the chamber. More precisely, there are three characteristic subpopulations of identified objects. Since antibody wash-off created an antibody concentration gradient increasing along the inflow direction in the chamber, CD4⁺ cells (green symbols) located at the “venting region” (10-11 mm) were well stained, while antibody deficiency in the “filling region” (0-1 mm) led to poor staining and the intensity of CD4⁺ cells located at the “filling region” (orange symbols) were much dimmer. In addition to the inhomogeneous staining pattern, the lower thresholds applied due to the smeared out population, caused the inclusion of false positive objects (pink open symbols) with slightly reduced intensities in the cell count. Those objects generally originated from the “venting region” where antibody accumulated after wash-off, as in such region the enhanced background intensity increased the background variation which led to erroneous identification of the objects during image analysis. Fig. 7.4e summarizes the intensities (APC and PerCP readout) of identified objects (i.e. CD4⁺ T-lymphocytes) at “filling” versus (vs.) “venting regions” of the counting chambers, which contain printed layers with different trehalose fractions.

We conclude that a printed gellan/trehalose (17%) layer is the optimal matrix to store and release antibody in the counting chambers for intense and homogenous immunostaining.

Fig. 7.4: a) Average intensities (PerCP vs. APC readout) of stained CD4⁺ T-lymphocytes (defined by manual gating from scatter plots b)-d) obtained from the counting chambers containing printed layers with varying trehalose fractions. Data points represent mean $\pm$ standard deviation ($n = 3$). Scatter plots and histograms of APC (red) and PerCP (blue) emission intensities from fluorescence images of the counting chambers containing: b) pure gellan (0%), c) gellan/trehalose (17%) and d) gellan/trehalose (33%). Black symbols represent all double (CD3 and CD4) positive cells (i.e. CD4⁺ T-lymphocytes) found in the entire image area. In scatter plot d), three subpopulations are specified. Orange symbols are CD4⁺ T-lymphocytes found in the "filling region". Green symbols are CD4⁺ T-lymphocytes found in the "venting region". Pink open circles are false positive objects found in the "venting region" due to antibody wash-off induced high background intensity and large background variation. e) Summary of average intensities (PerCP vs. APC readout) of stained CD4⁺ T-lymphocytes at "filling" and "venting regions". Data points represent mean $\pm$ standard deviation ($n = 4$).

7.3.4 Validation of CD4 counts obtained by image cytometry

Flow cytometry is considered the gold standard in CD4 counting. To validate the sensitivity and specificity of our CD4 counting approach, a comparison of CD4 counts obtained with flow cytometry and our image cytometry was performed. Blood samples with 7 different concentrations of leukocytes were prepared. As can be seen in Fig. 7.5, the results obtained from the two approaches correlate well over a large

range of cell concentrations (~ 5 -1000/ μL).

Fig. 7.5: Comparison between CD4⁺ T-lymphocyte concentrations ($\#/\mu\text{L}$) of whole blood samples and leukocyte depleted samples from healthy donors, measured using our image cytometry approach and with flow cytometry. For each sample with a given leukocyte concentration, 4 separate counting chambers were used (individual cross) and 3 separate flow cytometry assays are performed. The averages of the flow cytometry results were used to plot the data.

7.4 Conclusion

Here, we demonstrate improved CD4 counting chambers with on-chip sample preparations for POC diagnostics. Automated chamber fabrication including matrix and frame printing greatly reduces the need for manual labor, while increasing the chamber reproducibility, compared with the previous chamber assembly process. The composite gellan/trehalose material proves to be a suitable matrix that preserves the fluorophore of the labeled antibody during storage and achieves intervention-free on-chip immunostaining after sample filling. The CD4 counts obtained from our image cytometry correlate well with the results from standard flow cytometry. This automated and inexpensive fabrication process together with the simplicity of on-chip sample preparation as well as the accuracy of the determined cell counts, exactly fulfills the requirement for microfluidic devices in POC diagnostic applications.

In addition to this specific application of CD4 counting, we believe that the printing technique used for chamber fabrication in combination with the concept of

on-chip sample preparation can be used as a versatile approach to realize a large variety of immunostaining based cytometry assays.

References

1. D. Y. Stevens, C. R. Petri, J. L. Osborn, P. Spicar-Mihalic, K. G. McKenzie and P. Yager, *Lab Chip*, 2008, 8, 2038-2045.
2. M. Hitzbleck, L. Gervais and E. Delamarche, *Lab Chip*, 2011, 11, 2680-2685.
3. F. Brandl, F. Kastner, R. M. Gschwind, T. Blunk, J. Tessmar and A. Gopferich, *J. Control. Release*, 2010, 142, 221-228.
4. S. Ganta, H. Devalapally, A. Shahiwala and M. Amiji, *J. Control. Release*, 2008, 126, 187-204.
5. J. K. Oh, R. Drumright, D. J. Siegwart and K. Matyjaszewski, *Prog. Polym. Sci.*, 2008, 33, 448-477.
6. M. Molina, M. Asadian-Birjand, J. Balach, J. Bergueiro, E. Miceli and M. Calderon, *Chem. Soc. Rev.*, 2015, 44, 6161-6186.
7. M. Tijero, R. Diez-Ahedo, F. Benito-Lopez, L. Basabe-Desmonts, V. Castro-Lopez and A. Valero, *Biomicrofluidics*, 2015, 9, 044124.
8. S. Ramachandran, E. Fu, B. Lutz and P. Yager, *Analyst*, 2014, 139, 1456-1462.
9. M. Beck, S. Brockhuis, N. van der Velde, C. Breukers, J. Greve and L. W. M. M. Terstappen, *Lab Chip*, 2012, 12, 167-173.
10. M. Mortato, L. Blasi, G. Barbarella, S. Argentiore and G. Gigli, *Biomicrofluidics*, 2012, 6, 044107.
11. R. R. Niedl and C. Beta, *Lab Chip*, 2015, 15, 2452-2459.
12. D. Puchberger-Enengl, C. Krutzler, F. Keplinger and M. J. Vellekoop, *Lab Chip*, 2014, 14, 378-383.
13. E. Jang, S. Kim and W. G. Koh, *Biosens. Bioelectron.*, 2012, 31, 529-536.
14. Z. Zhao, M. A. Al-Ameen, K. Duan, G. Ghosh and J. F. Lo, *Biosens. Bioelectron.*, 2015, 74, 305-312.
15. V. N. Luk, L. K. Fiddes, V. M. Luk, E. Kumacheva and A. R. Wheeler, *Proteomics*, 2012, 12, 1310-1318.
16. M. Hitzbleck and E. Delamarche, *Chem. Soc. Rev.*, 2013, 42, 8494-8516.
17. E. Tekin, P. J. Smith and U. S. Schubert, *Soft Matter*, 2008, 4, 703-713.
18. B. J. de Gans, P. C. Duineveld and U. S. Schubert, *Adv. Mater.*, 2004, 16, 203-213.
19. K. Abe, K. Kotera, K. Suzuki and D. Citterio, *Anal. Bioanal. Chem.*, 2010, 398, 885-893.
20. J. T. Kirk, G. E. Fridley, J. W. Chamberlain, E. D. Christensen, M. Hochberg and D. M. Ratner, *Lab Chip*, 2011, 11, 1372-1377.
21. T. Fujie, A. Desii, L. Ventrelli, B. Mazzolai and V. Mattoli, *Biomed. Microdevices*, 2012, 14, 1069-1076.
22. H. Sirringhaus, T. Kawase, R. H. Friend, T. Shimoda, M. Inbasekaran, W. Wu and E. P. Woo, *Science*, 2000, 290, 2123-2126.
23. X. Zhang, D. Wasserberg, C. Breukers, L. W. M. M. Terstappen and M. Beck, *Analyst*, 2016, 141, 3068-3076.
24. C. A. Schneider, W. S. Rasband and K. W. Eliceiri, *Nat. Methods*, 2012, 9, 671-675.
25. J. K. Kaushik and R. Bhat, *J. Biol. Chem.*, 2003, 278, 26458-26465.
26. N. K. Jain and I. Roy, *Protein Sci.*, 2009, 18, 24-36.

27. J. L. Cleland, X. Lam, B. Kendrick, J. Yang, T. H. Yang, D. Overcashier, D. Brooks, C. Hsu and J. F. Carpenter, *J. Pharm. Sci.*, 2001, 90, 310-321.
28. T. Coviello, P. Matricardi, C. Marianecci and F. Alhaique, *J. Control. Release*, 2007, 119, 5-24.
29. P. Matricardi, C. Cencetti, R. Ria, F. Alhaique and T. Coviello, *Molecules*, 2009, 14, 3376-3391.
30. H. Hegyi and M. Gerstein, *J. Mol. Biol.*, 1999, 288, 147-164.
31. G. G. Chang and H. J. Lee, *J. Biochem. Biophys. Methods*, 1984, 9, 351-355.
32. O. V. Stepanenko, V. V. Verkhusha, V. I. Kazakov, M. M. Shavlovsky, I. M. Kuznetsova, V. N. Uversky and K. K. Turoverov, *Biochemistry*, 2004, 43, 14913-14923.
33. D. J. Jang and M. A. Elsayed, *Proc. Natl. Acad. Sci. U. S. A.*, 1989, 86, 5815-5819.
34. S. M. Akbar, K. Sreeramulu and H. C. Sharma, *J. Bioenerg. Biomembr.*, 2016, 48, 241-247.
35. A. M. Lammerta, S. J. Schmidta and G. A. Dayb, *Food Chem.*, 1998, 61, 139-144.

Summary and Outlook

The success of point-of-care (POC) diagnostic tests strongly relies on the automated sample preparation without manual intervention and external equipment. This thesis presents the development of an on-chip sample preparation concept to achieve “InstantCount” concept: immunophenotyping and enumeration of CD4 positive (CD4⁺) T-lymphocytes (CD4 counting) in whole blood for POC. In this concept, a variety of hydrogel matrices, with embedded fluorophore-labeled antibodies as staining agents, were integrated into cell counting chambers using different techniques. The on-chip antibody release from the hydrogel matrix in a flow chamber, which mimics the inflow of whole blood to the counting chamber, was studied and the parameters that govern the release kinetics were identified. The in-depth understanding of the on-chip release gained in the flow chamber can be translated to tune the distribution of antibody in the counting chamber for homogenous on-chip staining.

To study the release kinetics, a real-time and *in situ* fluorescence imaging system to monitor the release of antibody in flow chambers was developed. In Chapter 2, allophycocyanin labeled α CD3 IgG (APC- α CD3) was used to demonstrate that the fluorescence intensity is a valid signal to quantify antibody release. To expand the application, the optical setup can be upgraded with additional LEDs and filters to enable the measurement of the release kinetics of other fluorescent molecules with different excitation and emission wavelengths.

In Chapter 3, the kinetics of on-chip antibody release from (sub-) μ m-thick gelatin layers in the flow chambers was studied. The measured release was revealed to comply with a diffusion controlled model and the layer thickness dependent release time was determined. By varying the layer preparation conditions: temperature, humidity and drying time; we were able to influence the material properties of the layer, thereby tuning the release time of antibody. There was a trend observed between the release time obtained from the flow chamber and the antibody distribution throughout the counting chamber: the longer the release time, the more

homogeneous the antibody distribution, and further the more uniform the cell staining. This correlation proves that detailed knowledge of the release kinetics in the flow chambers can be translated into the performance of sample-reagent mixing in the counting chambers. The concept of this study can be extended to investigate on-chip release of a variety of reagents (i.e. lysis agents, fluorescent dyes, enzymes and drugs) from hydrogel matrices. The understanding of the release kinetics of these reagents can be used as guide to tune the sample-reagent mixing in a multitude of microfluidic devices and optimize on-chip sample preparation for many biological assays.

In Chapter 4, inkjet printing was employed to deposit gelatin layers in the counting chambers to release fluorophore-labeled antibody on demand in order to achieve optimal staining for image cytometry. The active antibody release was first verified in the flow chambers: the initial release was delayed by applying an appropriate maturation protocol to printed layers, in which the antibody was trapped due to the excessive crosslinking; on-demand release was instantaneously triggered using temperature switch to dissolve the entire layer, thereby achieving complete release. The application of active release scheme in the counting chambers (with stopped-flow configuration) fulfills the exact criteria (delayed release to prevent antibody wash-off during blood inflow and subsequent complete release to attain rapid and homogenous cell staining) for on-chip sample preparation in our CD4 counting concept. In addition to immunostaining based image cytometry, this method for reagent release on demand can also be used in many other biological assays, requiring temporally or spatially controlled release for complex sample-reagent mixing schemes.

It is commonly acknowledged that CD4 counting in low-income and resource-limited settings is only practical and affordable, if all manual sample preparation is avoided and data analysis is fully automated, while, at the same time, the tests need to be fabricated at very low cost. In Chapter 5, the extension of the previous research to low-cost fabrication of CD4 counting chambers via drop-on-demand printing techniques is presented. Printing protocols to fabricate

chamber compartments by dispensing mono-sized microbeads, suspended in UV-curable glue, using a piezo-actuated nanoliter dispenser and deposit hydrogel/antibody layers for on-chip reagent storage and sample preparation, by inkjet printing were developed. These fully printed CD4 counting chambers were successfully tested with blood from healthy donors and patient samples. The CD4 counts obtained using this image cytometry method agreed well with the result from the gold standard, flow cytometry. The extremely simple concept and low-cost production method of the CD4 counting assay presented here, has a great potential to be translated to many other cytometric assays which rely on cell staining (via fluorophore-labeled antibody or nuclear dyes). The concept gains great flexibility and speed via the use of printing techniques for fabrication and can, therefore, be adapted to rapidly test new cell counting assays.

Due to the ongoing maturation of gelatin, continuous changes in release kinetics of antibody from gelatin layers results in poor reproducibility and short shelf life of the counting chambers containing gelatin layers. To overcome this limitation, in Chapter 6 the identification of alternatives for gelatin as a matrix material for the CD4 counting chambers was carried out. Eleven different polysaccharides were tested as potential matrix materials. They were divided into 3 groups: pure polysaccharides, polysaccharide blends and ionically crosslinked polysaccharide blends. Among these candidates, only gellan meets all criteria required of a matrix material to achieve complete as well as homogeneous immunostaining within 30 min in the counting chambers and shows long-term stability. The screening protocol in this chapter can be extended to test a much broader range of materials (commonly used synthetic polymers and bio-materials in tissue engineering and *in vivo* drug delivery) for the identification of even better matrix candidates.

In Chapter 7, the development of fully automated fabrication of the CD4 counting chambers containing a gellan/trehalose matrix, an alternative to gelatin, for on-chip antibody preservation and release is described. The fabrication of the counting chambers and the deposition of matrix material were executed in an automated

manner. Due to the simple and low-cost fabrication by drop-on-demand printing, the time and skill required for the fabrication of CD4 counting chambers is greatly reduced; meanwhile chamber reproducibility is significantly improved. Gellan/trehalose layers showed the potential for good on-chip antibody preservation before use and simple intervention-free on-chip immunostaining after sample inflow. The CD4 count obtained from automated image analysis correlated very well with the result of gold standard, flow cytometer. This automated and inexpensive fabrication process together with the simplicity of on-chip sample preparation and accurate cell count exactly fulfills the requirement for microfluidic devices in POC diagnostic applications.

In conclusion, a new concept by incorporating drug delivery system in microdevices to achieve on-chip sample preparation for immunostaining based cell identification and enumeration was developed. Passive and active antibody release schemes from differently prepared gelatin matrices were successfully demonstrated in the flow chambers and the acquired knowledge provides valuable guidance to tailor the antibody distribution in the counting chambers for homogenous staining and accurate cell count. In addition to gelatin, polysaccharide matrices were tested in this concept and gellan was found to be a promising alternative matrix with prolonged stability. In addition, the fabrication hydrogel/antibody integrated counting chambers in a fully automated manner was achieved. The new counting chambers, fabricated by low-cost/high-throughput drop-on-demand printing technique, enable complete on-chip sample preparation, thus fulfilling all the basic criteria for a practical and affordable CD4 counting assay in resource-limited settings. The current study narrows down the scope by limiting the release matrix to hydrogel material and focusing only on immunostaining based cell counting, which only requires one-step mixing in the sample preparation. However, by exploring more in material science and taking advantage of novel material dispensing techniques, this concept has the potential to achieve multistep bioassays requiring sequential reagent release and multiplex biosensing requiring spatial reagent release. Integrating a multilayer structured matrix, which allows layer-by-layer release in microdevices would be attractive for complex bioassays, in particular the analysis of intracellular molecules from a certain

type of cells. Defined patterning of different matrix/reagent patches in microdevices enabling compartmentalized release will provide a solution for high-throughput biosensing.

Samenvatting

Het succes van point of care (POC) diagnostische testen hangt sterk af van geautomatiseerde monster voorbehandeling zonder enige handmatige interventie en externe apparatuur. Deze scriptie beschrijft de ontwikkeling van een concept voor een “on-chip” monster voorbehandeling dat het mogelijk zou moeten maken om een bloed monster te kunnen immunofenotyperen en bijvoorbeeld de hoeveelheid CD4⁺ T-lymfocyten te bepalen in een POC setting. In dit concept zijn een aantal hydrogels geïntegreerd in cel telkamers met behulp van verschillende technieken. In de hydrogels zitten fluorescent gelabelde antilichamen ingebouwd die nodig zijn voor het “herkennen” van de cellen. Het “on-chip” antilichaam afgifte mechanisme uit de hydrogel matrix in een stromingskamer, waarmee de instroom van een bloed monster in een telkamer nagebootst, werd bestudeerd en de parameters welke de afgiftekinetiek regelen werden geïdentificeerd. De kennis die hieruit werd vergaard kan worden gebruikt om de verdeling van antilichamen, in de uiteindelijke telkamer, te optimaliseren voor een homogene “on-chip” verdeling van de fluorescent gelabelde antilichamen voor een accurate immunofenotypering van de cellen.

Om de afgiftekinetiek te bestuderen is er een real-time *in situ* fluorescentie imaging systeem ontwikkeld, om de afgifte te kunnen monitoren. In hoofdstuk 2 werd er gebruik gemaakt van APC gelabelde anti-CD3 antilichamen om te demonstreren dat de intensiteit van het fluorescentie signaal kan worden gebruikt om de antilichaam afgifte te kwantificeren. Om de applicatie mogelijkheden verder te verbreden kan de optische opstelling worden uitgebreid met extra LED's en filters die het mogelijk te maken de afgiftekinetiek te bepalen van andere fluorescent gelabelde moleculen met andere excitatie en emissie golflengtes.

In hoofdstuk 3 is de “on-chip” antilichaam afgiftekinetiek in stromingskamers met een (sub-) μm -dikke gelatine laag bestudeerd. De gemeten afgifte komt overeen met een diffusie gecontroleerd model en de laag dikte afhankelijke afgifte tijd werd bepaald. Door de laag preparatie condities te variëren (zoals de temperatuur,

vochtigheid en droogtijd) hebben we de materiaal eigenschappen van de laag kunnen beïnvloeden, waarmee we de afgifte tijd van het antilichaam hebben kunnen “fine tunen”. Een trend is aangetoond tussen de afgiftetijd en de antilichaam distributie over de telkamer: hoe langer de afgifte tijd, hoe meer homogeen de antilichaam distributie en zodoende ook een meer uniforme cel kleuring. Deze correlatie bewijst dat nauwkeurige kennis van de afgiftekinetiek in stromingskamers kan worden omgezet naar het functioneren van het mixen van reagentia en monster in telkamers. Het principe van deze studie kan worden uitgebreid richting onderzoek van “on-chip” afgifte van verschillende reagentia (zoals: lyserende reagentia, fluorescente kleurstoffen, enzymen en farmacologische componenten) uit hydrogel matrixen. Het principe van deze afgiftekinetiek kan worden gebruikt als een richtlijn voor monster-reagentia menging in een veelvoud van microfluidische opstellingen en voor het optimaliseren van “on-chip” monster voorbehandeling in vele verschillende biologische assays.

In hoofdstuk 4 is het gebruik van inkjet printen beschreven waarmee gelatine in lagen werd gedeponeerd in telkamers om fluorescent gelabeld antilichaam naar behoefte aftegeven om zo een optimale kleuring te verkrijgen voor cytometrische analyse. De actieve antilichaam afgifte werd eerst bevestigd in stromingskamers: de initiële afgifte werd vertraagd door een geschikt rijpingsprotocol voor geprinte lagen te gebruiken, waarbij het antilichaam werd ingevangen door overmatige crosslinking; antilichaam afgifte werd onmiddellijk getriggerd door gebruik van een “temperatuur schakeling” waardoor de gehele laag werd opgelost en zodoende een complete afgifte werd verkregen. De toepassing van een actieve afgifte in telkamers (met een zogeheten “stopped-flow” configuratie) vervult de exacte criteria (vertraagde afgifte om zo een antilichaam “wash-off” te voorkomen tijdens de inflow van bloed en de daaropvolgende complete afgifte, waarmee een snelle en homogene cel kleuring word verkregen) voor on-chip monster voorbehandeling met ons CD4 cel tel principe. Naast de geschiktheid voor antilichaam gebaseerde kleuring en daarop volgende cytometrische analyse van cellen is deze methode ook geschikt voor elke andere reagentia die op bepaalde momenten moet worden afgegeven (in het bijzonder als de

reagentia een temporaal en spatiaal gecontroleerde afgifte nodig hebben voor complexe monster-reagentia meng stappen).

Het is algemeen erkend dat CD4 cel tellingen in ontwikkelingslanden alleen praktisch en betaalbaar zijn als alle handmatige monster voorbehandeling wordt vermeden en data analyse volledig geautomatiseerd is. Daarnaast moeten de testen ook met zeer lage kosten worden geproduceerd. In hoofdstuk 5 wordt een verlengde van de voorgaande hoofdstukken bestudeerd, in het bijzonder richt het hoofdstuk zich op een laag kostende fabricage van CD4 telkamers doormiddel van “drop-on-demand” print technieken. Print protocollen werden ontwikkeld om kamer compartimenten te fabriceren waarbij zogeheten “monosized microbeads” werden gedisperseerd, gesuspenseerd in een UV-hardende lijm, doormiddel van een piezo nanoliter dispenser. Tevens werden hydrogel/antilichaam lagen gedeponeerd voor on-chip reagentia opslag en monster voorbereiding doormiddel van inkjet print technieken. Deze geprinte CD4 telkamers zijn met succes getest met bloed monsters van gezonde donoren alsook met patiënt monsters. De CD4 tellingen kwamen goed overeen met de gouden standaard, flow cytometrie. Het extreem simpele principe en de lage productie kosten van de CD4 tel assay heeft veel potentie om te worden omgezet voor andere cytometrische bepalingen die ook gebaseerd zijn op cel kleuring met behulp van fluorescent gelabelde antilichamen of celkern kleuringen. Ons principe is erg flexibel en snel om te zetten voor productie door het gebruik van print technieken, waarmee snel aanpassingen kunnen worden aangebracht aan een ontwerp om nieuwe cel bepalingen uit te testen.

Door de continue rijping van gelatine zijn er ook continue veranderingen met betrekking tot de afgiftekinetiek van antilichamen uit de gelatine lagen. Dit betekent dat er een relatief slechte reproduceerbaarheid is en een korte houdbaarheid voor tel kamers met gelatine lagen. Om deze tekortkoming te overwinnen zijn in hoofdstuk 6 verschillende alternatieven bestudeerd en gevonden om gelatine in deze tel kamers te kunnen vervangen. Elf verschillende polysachariden werden getest als potentiële vervangers. Ze werden verdeeld in 3 groepen: pure polysachariden, polysacharide

mengsels en ionisch gecrosslinkte polysacharide mengsels. Van al de kandidaat polysachariden bleek alleen gellan aan alle criteria te voldoen, het bleek een homogene kleuring mogelijk te maken in de telkamers binnen 30 minuten en het heeft een relatief lange houdbaarheid. Het gebruikte screenings protocol kan worden uitgebreid om een grotere hoeveelheid van alternatieve materialen te testen om zo mogelijk zelfs nog betere kandidaten te vinden dan gellan (men kan hierbij denken aan veel gebruikte synthetische polymeren en bio-materialen die worden gebruikt in tissue engineering en in *in-vivo* medicatie afgifte).

In hoofdstuk 7 is de volledig geautomatiseerde fabricage van CD4 telkamers met een gellan/trehalose matrix beschreven. Door de simpele en laag kostende fabricage (mogelijk gemaakt door “drop-on-demand” printen) worden de benodigde tijd en vaardigheid van de eindgebruiker drastisch gereduceerd, terwijl de reproduceerbaarheid significant wordt verbeterd. De gellan/trehalose lagen lieten zien de potentie te hebben om beter geschikt te zijn voor on-chip antilichaam houdbaarheid en een interventie-vrije on-chip cel kleuring. De vergelijking van CD4 tellingen tussen de door ons hier beschreven telkamers (en bijbehorende geautomatiseerde beeld analyse) en de flow cytometer (gouden standaard) lieten zien dat er een heel goede correlatie was tussen de beide methodes. De goedkope productie kosten, het geautomatiseerde fabricage proces, de simpele monster voorbehandeling en accurate cel telling vervullen precies de benodigde behoeftes die worden gesteld aan POC diagnostische apparatuur.

Samenvattend, in deze scriptie is de ontwikkeling van een nieuw concept beschreven, waarbij een medicatie aflever systeem met een zogeheten “micro device” werd gecombineerd. Dit systeem kan een monster volledig voorbehandelen voor de opeenvolgende kleuring van het monster door middel van fluorescent gelabelde antilichamen en daaropvolgend de identificatie en kwantificatie van de cellen in het monster. Passieve en actieve antilichaam afgifte methodes vanuit verschillende gelatine matrixen zijn gedemonstreerd en de vergaarde kennis geeft waardevolle inzichten met betrekking tot het op maat maken van de antilichaam distributie in de

telkamers om zo een homogene kleuring en accurate cel telling te verkrijgen. Naast gelatine zijn ook polysachariden getest als mogelijke matrixen. Gellan bleek het meest veelbelovend als gelatine alternatief. Daarnaast is het ook gelukt de telkamers volledig geautomatiseerd te produceren. Deze nieuwe telkamers, gemaakt doormiddel van een efficiënte drop-on-demand print techniek, maken het mogelijk om een volledige on-chip monster voorbehandeling te bewerkstelligen. Hiermee worden alle criteria vervuld om een praktische en goedkope CD4 cel tel bepaling mogelijk te maken in omgevingen met beperkte middelen (zoals ontwikkelingslanden). De scope van de hier beschreven studie is beperkt omdat we hebben gekeken naar een gelimiteerd aantal matrix alternatieven en we hebben ons alleen gericht op immunofenotypering gebaseerde cel tellingen (waarbij maar 1 mix stap nodig is voor de monster voorbehandeling). Echter kan de kennis die hier is vergaard in de toekomst worden verrijkt door te kijken naar andere matrixen en materialen en door te kijken naar nieuwere dispensie technieken. Hierdoor kan de techniek die hier beschreven is ook mogelijk worden gebruikt voor meer gecompliceerde assays waar meerdere afgifte en/of mengstappen gewenst zijn. Door een multilaagse gestructureerde matrix in micro devices in te bouwen kan mogelijk een afgifte per laag worden gerealiseerd, waardoor meer gecompliceerde cel studies mogelijk worden en er bijvoorbeeld kan worden gekeken naar intracellulaire moleculen. Door gebruik te maken van een gedefinieerd patroon van verschillende reagentia/matrix plekken in micro devices (waardoor gecompartmenteerde afgifte kan worden gerealiseerd) kan deze techniek ook mogelijk een oplossing bieden voor high-throughput biosensing.

Acknowledgement

At the end of this thesis, I would like to thank all the “crew members” who company with me to finish the voyage of my PhD. It is a rather long and challenging expedition. Without your support and encouragement, I could neither sail this far as a “Captain” alone nor embrace the sunshine on the “New World” after being through all the sea storms behind.

First of all, I greatly appreciate my promoter, Leon. You are an excellent “Steersman”. The first 2-year of my PhD was chaotic and the course of the project was severely deviated. Right at that moment, you decisively arranged more frequent meetings between us to guide me out the whirlpool of experiment details. Your sense of the project progress in a large picture always pointed out the direct path to follow. Thanks to this not-too-late rescue, I gained sufficient output in the last 2 and half years, which surprises both you and me.

I would like to express my sincere gratitude to my supervisor, Markus. You are the “Chief Engineer”, the most knowledgeable guy on board. In addition to maintaining everything in good conditions, you were even more curious and positive on my failed experiments and striving to come up with an idea on how we can benefit from these unexpected phenomena. Detail-oriented spirit and problem-solving ambition were what I learned from you during every discussion between us. Although it might be time-consuming (the “half-day” long discussion turned every Monday into the least “productive” day of the week.), it was definitely worthwhile to train me to think as a real scientist. Sometimes the training of logic thinking was also useful when we were arguing football issues between BVB (my side) and Bayern.

Dodo, my respect “Chief Deck Officer”, was doing an amazing job to ensure the smooth operation in “InstantCount” project. You are always kind to answer all my technical inquiries promptly. I learned a lot from you on how to become an organized scientist. I am deeply impressed by your methods of archiving experiment records of

countless projects at the same time in the lab journal with labels of all your “hated” colors and storing results systematically in a Gigabyte-large Origin files which require a super computer to initialize. In addition to our intensive collaboration at work, we also had plenty of pleasant chats about huskies, in particular your adorable Cosar and Comid, at coffee breaks. The moments were so enjoyable that I started to change myself from a coffee-allergic guy to a frequent Starbucks customer.

I am also very grateful to all the other InstantCount teammates. Christian, you are a genius “Electro-technical Officer” who gave the birth to our proprietary ICARUS printing system, which is now on the cover of this thesis as my forever memory. Joost vD, as a “First Engineer”, you kept broadening my horizons on new stuff and fancy technology, especially the drone you created by yourself. Bridgette and Èlia, you are always friendly and showed incredible caring on the teammates which made me feel very warm in our united team.

Special thanks will go to Ivan and Hoon. I am very lucky to be surrounded in a very relaxed and funny office environment. I have to say, you guys fulfilled extraordinary roles of “First Officers”, that is to make the “Captain” happy. Ivan, you are a man full of humor sense that you can easily make jokes on everything. You also generously offered me a great hand to sort out a lot of daily life issues, most importantly you helped me to pick the first car of my life. It never fails me and I hope it can still stick to its duty to offer me a safe trip here on my Big Day. Hoon, your settlement in office extended the happy hour tradition. In the last few months, you tried to relieve my stress and cheer me up by making fun of the way I worked every day (endless text correction, continuous figure redrawing, infinite deadline reschedule). I really appreciate those funny jokes which refreshed me from the tedious work.

Thanks to all the other MCBP group members for the great time we spent together. Ingrid, I was always welcome in your office to seek any help and you were so professional that there was no single question that confused you. FC MCBP teammates:

Joost S (you were my valued running advisor when I made my first attempt to half-marathon.), Michele, Ivan, Niels, Fikri, Rick and Agustin (although we just played a few times, I was impressed that you were as good as Chicharito.), it was great experience to play with you and I felt very proud to witness our 5-men indoor team growing from nothing to a fantastic squad. During the last 5 years, there were also a lot of good memories with you: Afroditi, Anouk, Arjan, Aufried, Barbara, Frank, Guus, Kiki, Lisa, Pepijn, Sanne (Ancient mystery disclosure: I was your Sinterklaas who sent calendar at the gift exchange organized by Bridgette back then), Stef and Yoonsun.

In addition to fully concentrating on the voyage, I enjoyed a lot in my leisure time with the Chinese friends I met in Enschede and later in Utrecht. 靳鹏, 林龙泉, 海荣, 刘尧和叶良在我初来乍到之时, 给予了我莫大的关怀, 让我迅速适应了这里的生活。醒吾和海山在我努力完成论文最苦恼的阶段, 帮我缓解很多的积郁和烦闷。感谢你们在我最困难的时候帮助我。在 Enschede 的丰富生活离不开一群志同道合的朋友: 蓝天, 杜莹, 王荣, 范佑文, 珺文, 嘉慧, 陆洲, 王毅, 刘爱洁。搬家后, 在 Utrecht 又有幸结识一群热爱足球的兄弟。在此先感谢 UUFC 的所有队员, 正是因为这个集体的红火, 我的照片才有幸第一次登上报纸 Utrechtse Sports Krant。最后特别感谢思毅队长和瑞学队副的接纳, 以及志雄, 宏凯, 娄博, 陈晨和桃子在长期的比赛和训练中给予的关照。

I am especially thankful to my parents and my grandparents. 母亲和父亲虽然都不是知识分子, 在学业和事业上已不能再教导我了, 但是母亲宽厚待人和父亲一丝不苟的品行却仍是我一生学习的榜样。感谢你们的默默支持和深切教诲, 我才有了今天的成绩。祖父一直希望我读书破万卷, 如今我修完博士学位离不开您孜孜不倦的鼓励。祝愿祖父母和外祖母身体健康, 继续见证我的成长。

In the end, I could not find words to express how much I am grateful to my beloved, Yujie. 十一载, 白驹过隙。从刚上大学的青涩少年, 到如今已近而立之年, 你见证了我许多的沉浮, 从始至终你都相信我是值得信赖和依靠的。在很多迷茫的时候, 你没有躲避眼前的困苦, 没有惧怕未来的艰险, 你一直在我身边, 包容我宽慰我。展望未来, 我想到的只是一句话: 你若不离不弃, 我定生死相依。

List of publications

Journal Articles

X. Zhang, D. Wasserberg, C. Breukers, L. W. M. M. Terstappen and M. Beck, *Analyst*, 2016, 141, 3068-3076.

X. Zhang, D. Wasserberg, C. Breukers, L. W. M. M. Terstappen and M. Beck, *ACS Appl. Mater. Interfaces*, 2016, 8, 27539–27545.

X. Zhang, L. W. M. M. Terstappen and M. Beck *Point-of-Care Diagnostics – New Progresses and Perspectives* (Book Chapter), *IAPC-OBP* (In Press).

Conference contributions

X. Zhang, D. Wasserberg, C. Breukers, L. W. M. M. Terstappen and M. Beck, “Kinetics of antibody release from gelatin layers for on-chip sample preparation” *4th European Conference on Microfluidics (μFlu’14)*: Limerick, Ireland, Poster presentation.

X. Zhang, J. van Dalum, D. Wasserberg, C. Breukers, L. W. M. M. Terstappen and M. Beck, “Reagent release from hydrogel-coated cell counting chambers for on-chip sample preparation” *30th Congress of the International Society for Advancement of Cytometry (Cyto 2015)*: Glasgow, Scotland, Poster presentation.

J. van Dalum, X. Zhang, D. Wasserberg, C. Breukers, L. W. M. M. Terstappen and M. Beck, “Low-cost point-of-care diagnostics using printed cell counting chambers” *20th International Conference on Miniaturized Systems for Chemistry and Life Sciences (MicroTAS 2016)*: Dublin, Ireland, Poster presentation.

About the author

Xichen Zhang was born on 25th April 1987 in Wuhan, China. He received the Bachelor Degree in Applied Chemistry from Wuhan University (China) at 2009. During the bachelor study, he was engaged in the research project to develop polysaccharide based *in vitro* drug delivery system and contributed to two journal publications (*Int. J. Pharm.*, 2008, 357, 15-21; *Langmuir*, 2009, 25, 11720–26). In 2010, he received the Master Degree in Medical and Molecular Biosciences from Newcastle University (UK) by completing the Master thesis project in developing redox fluorescent biosensors for the measurement of reactive oxygen species in stimulated macrophages. Following that, he was appointed as a Project Officer by a research laboratory in Nanyang Technological University (Singapore), where he carried out the screening of anti-breast cancer compounds using hydrogel based 3D cell culture models and animal models. After that, he started to pursue his Ph.D. in University of Twente (the Netherlands) since 2012. For the last 4 and half years, he participated the European Research Council financed “InstantCount” project to develop image cytometry technology for point-of-care blood diagnostics, in particular for CD4 counting assay. In the project, he was specialized in studying hydrogel based controlled antibody release in microfluidic devices and translating the discovery to enable intervention-free on-chip sample preparation for cytometric assays in whole blood.

“Sometimes in life there is that moment when it's possible to make a change for the better. This is ***ONE*** of those moments.”

Elizabeth Glaser

ISBN 978-90-365-4274-6