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Automated Image Analysis in Fluorescence Microscopy

Habilitation Thesis
Collection of Articles

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Abstract

After completion of human genome project in 2003 a significant effort is put into research of structure and especially function of proteins in both cells and whole organisms. The most suitable technique to carry out this research in particular in living specimens is fluorescent microscopy. There are two main directions in fluorescence microscopy: (1) high-resolution imaging focused on observing details, typically in multi-dimensional data, and (2) high-throughput imaging focused on analyzing massive image data sets. Both directions raise challenges for automated image analysis.

The main goal of this thesis is to summarize my principal achievements in the area of automated image analysis in fluorescence microscopy and to show context and relations between the works. The achievements have been obtained in three fields: (1) automation of confocal microscopy, (2) automation of micro-axial tomography, and (3) image analysis in high-content screening. From the methodological point of view, this comprises novel methods for (1) point-based image registration of 3D confocal images of living cells and micro-axial tomography data, (2) chromatic aberration correction using polynomial functions, (3) object tracking with split and merge events based on combining optimal assignment problem and maximal flows, (4) cell nucleus segmentation based on a combination of a gradient thresholding scheme and mathematical morphology operators suitable for high-content screening, (5) quantification of the level of assembly or disassembly of cellular phenotypes based on mathematical morphology, and (6) spot detection in 3D confocal data based on extrema dynamics measurements. All presented methods have successfully been applied in real biological problems.

The thesis is written as a commentary to a collection of 14 peer-reviewed journal articles and 4 peer-reviewed conference papers. My percentage contribution for each paper is estimated and included in the thesis as well as a detailed description of my work. My percentage contribution to the papers ranges from 3% to 70%. Average percentage contribution to the papers is about 30%.

Abstrakt

Po té, co byl v roce 2003 dokončen projekt sekvenace lidského genomu, je dalším velkým výzkumným cílem pochopení vztahu struktury a funkce jednotlivých proteinů jak na buněčné úrovni tak i na úrovni celých organizmů. Nejvhodnější technikou k provádění tohoto výzkumu, a to zejména na živých buňkách, je fluorescenční mikroskopie. Zde lze historicky vypozorovat dva základní směry vývoje: (1) mikroskopie s vysokým rozlišením, která typicky produkuje vícedimenzionální obrazy a (2) tzv. "high-throughput" mikroskopie (HTM), která produkuje velké množství obrazů. V obou případech se jedná o velké množství dat, a proto je nutné používat automatickou analýzu obrazu.

Hlavním cílem této práce je sumarizovat nejdůležitější výsledky mojí vědecké práce v oblasti automatizované analýzy obrazů pořízených fluorescenčními mikroskopy a ukázat kontext práce a vztah mezi výsledky. Nejdůležitějších výsledků jsem dosáhl v těchto třech oblastech: (1) automatizace konfokální mikroskopie, (2) automatizace mikro-axiální tomografie a (3) obrazová analýza v oblasti HTM. Z metodologického pohledu se jedná o tyto nové metody: pro (1) registraci 3D konfokálních obrazů živých buněk a pro registraci dat z mikro-axiálního tomografu, které jsou založeny na extrakci významných bodů, (2) korekci barevné vady s využitím polynomiálních funkcí, (3) sledování pohybu dělících se a spojujících se objektů pomocí kombinace optimálního přiřazení v bipartitních grafech a maximálního toku v grafu, (4) segmentaci buněčných jader, která je založena na kombinaci gradientního prahování a metod matematické morfologie, jenž je vhodná pro screening pomocí HTM, (5) kvantifikaci míry kompaktnosti nebo difuzivity struktur uvnitř buňky s využitím matematické morfologie a (6) detekci malých objektů ve 3D konfokálních datech, která je založena na měření dynamiky extrémů v obraze. Všechny popsané metody byly úspěšně použity v reálných biologických aplikacích.

Práce je koncipována jako soubor čtrnácti časopiseckých a čtyř sborníkových prací, které prošly řádným recenzním řízením a byly publikovány na mezinárodním fóru. U každé práce je uveden procentuální odhad mého podílu na jejím vytvoření včetně detailního popisu vlastního příspěvku. Můj podíl se pohybuje v rozmezí od 3% do 70%. Průměrný podíl na jeden příspěvek je okolo 30%.

Acknowledgments

My warmest thanks are due to all co-authors of the included papers. It was a real pleasure to work with them on the exciting problems. In particular, I want to thank Michal Kozubek and Karl Rohr. Michal is the group leader of the Center of Biomedical Image Analysis (CBIA) at the Faculty of Informatics, who gave me the opportunity to work in his group, where I have learned a lot especially in my early scientific career. Karl is the group leader of the Biomedical Computer Vision (BMCV) Group at the German Cancer Research Center and the University of Heidelberg, who offered me a post-doc position in his group, where I got opportunity to work on many cutting edge life science problems, and who significantly influenced my way of thinking and working. I want also thank all my current or former colleagues from CBIA and BMCV groups. Last but not least, I would like to thank my family and especially my wife for her never-ending support.

In Fontainebleau, April 2012 Petr Matula

Contents

Part I

Commentary

Chapter 1

Introduction

The presented habilitation thesis consists of a collection of fourteen peer-reviewed journal papers and four peer-reviewed conference papers. This introduction presents the overall motivation, the context of the work, and the state of the art in the area. The next chapters provide a brief overview of the methods and results in my main research and development fields together with the description of my contributions and they also highlight the relations among individual papers.

1.1 Motivation and Context

The human genome project has identified approximately 20,000–25,000 human genes (DNA coding sequences). Each gene codes a unique protein[1] that performs a specific function in a cell. The cell is the smallest functional unit of life. However, the function of a vast majority of proteins is unknown[2]. The desire to understand the role of genes and proteins on the cellular level as well as on the higher levels such as tissues, organs and whole organisms is the current driving force of many life sciences. For example, building mathematical models that describe structure and function of biological systems by means of their reverse engineering[3] is the main topic of an interdisciplinary field called *systems biology* [35, 37, 13]. In order to draw relevant biological conclusions and to build sound mathematical models a close *interdisciplinary collaboration* of experts from many different fields is necessary, in particular molecular and computational biology, biophysics, bio-informatics, statistics, mathematics, and computer science.

Cytometry is a technologically oriented science focused on obtaining measurements on molecular or cellular targets by means of flow or image cytometry [85, 82]. While *flow cytometry* [74] is suitable for obtaining a large number of basic measurements (such

[1] Proteins are the most important macromolecules in living systems and serve crucial functions in essentially all biological processes [3]. They function as catalysts, they transport and store other molecules such as oxygen or other proteins, they provide mechanical support and immune protection, they generate movement, they transmit nerve impulses, and they control growth and differentiation.

[2] We know the letters, we know the words, but we do not understand the meaning.

[3] i.e. systematic perturbation of the biological system (biologically, genetically, or chemically); monitoring the gene, protein, and informational pathway responses; integrating these data for the mathematical modeling.

as cell size, presence or absence of proteins of interest, DNA content) on cells in liquid suspensions, *image cytometry* is used for advanced measurements (such as morphological measurements or spatial and dynamic relations of proteins and genes) based on microscopy. Electron as well as optical microscopy are widely used for morphological studies, but optical microscopy is a unique technique used in live cell imaging.

Fluorescence microscopy is a technique based on optical microscopy, which is of a critical importance in modern life sciences. It uses fluorochromes, which are chemical compounds that can re-emit light upon light excitation, for specific visualization of objects of interest. This allows in combination with staining techniques (such as FISH—Fluorescence In Situ Hybridization[4] and/or immunofluorescence[5]) or genetic modifications[6] highly specific observation and measurement of fluorescent signal even on a single molecule level in both fixed and live cells.

1.2 History and State-of-the-Art

There has been two main parallel trends in the development of fluorescence microscopy: (1) *resolution improvement* to obtain images containing more details and (2) *automation* to acquire and analyze more data. There are many resolution improvement techniques such as confocal microscopy, 4Pi microscopy, structured illumination microscopy, micro-axial tomography[7], see [30, 33] for a review. The most wide-spread resolution improvement technique is confocal microscopy [66] where the out-of-focus light is blocked before the detector[8] and 3D optical sections of a sample can be obtained. The first automated microscopes appeared in late 1970s and became commercially available to biologists in late 1990s when the first high-content screening platform was built [25, 83]. At that time when fluorescence staining techniques matured and automation components were commercially available, research groups began to automate fluorescent microscopes to perform specific tasks, e.g., FISH-dot counting [60, 63, 40]. One of them was built by Michal Kozubek et al. at the Institute of Biophysics in Brno [40]. This automated microscope has been extended by a Nipkow disk based confocal unit to permit combination of wide-field[9] and confocal modes [39]. While developing this combined automated microscope many different image analysis problems appeared. I worked especially on the correction of chromatic aberrations (Section 2.1). Later, a new microscope supporting high-resolution live cell imaging was constructed [42], and I have worked on image analysis problems related to object tracking and image registration (Section 2.3). Nowadays, automated microscopes are offered by the main microscope manufactures and wide-field as well as confocal screening platforms[10] are

[4] Visualization of DNA sequences—genes

[5] Visualization of antibodies—proteins

[6] Virtually any protein can be modified to exhibit fluorescence without changing its primary function. The invention and development of green fluorescent protein (GFP) was awarded the Nobel Prize for chemistry in 2008 to Osamu Shimomura, Martin Chalfie, and Robert Y. Tsien.

[7] Several results included in this thesis relate to this resolution improvement technique (see Section 3.1).

[8] Pinholes in CLSM (Confocal Laser Scanning Microscopy) or spinning disks in Nipkow-disk confocal microscopy.

[9] non-confocal

[10] A screening platform differs from an automated microscope in the integration and automation of the whole analytical process. Often, the screening platforms produce and analyze a larger number of images at lower resolution than automated microscopes.

commercially available [28, 84]. The commercially available screening platforms are build for certain types of assays often designed to meet the needs of drug discovery [4], but they also typically provide an option to save the raw images and to analyze them in an external software package. External software packages[11] and/or involvement of image analysis experts is necessary, in particular, for specialized and complex problems often related to live cell imaging [90].

From the point of view of image analysis there exists no universal solution that could be applied to any biological problem. Based on own experience, even a small change in the biological protocol or in the image acquisition process can dramatically change the image analysis problem to solve. Similarly, small misunderstanding of the biological problem by an image analysis expert or misunderstanding of the limitations and assumptions of image analysis method by a biologist especially in the results interpretation phase can have serious consequences. Therefore close interaction, good communication between all engaged persons, and good problem understanding are crucially important to find the right solution. Nevertheless, there are some typical problems that appear often in the field, for example, cell nucleus segmentation, whole cell segmentation, or small spot detection. However it is very difficult to compare different methods unless they are tested on the same data and under the same conditions and even then it can be difficult to decide what approach is better than the other without knowing the real application (see e.g., [77]).

Recently, several survey papers have been published discussing typical or common image analysis methods in automated fluorescence microscopy [2, 31, 70]. In the sequel, a brief overview of the areas related to the main topics of the thesis are provided, namely image correction, segmentation, classification, registration, and tracking.

Image correction It is common to correct images before any further processing, but it should be done with care and only if one knows the source of distortion. The most common type of correction is the correction of inhomogeneities in the fluorescence signal [47, 58]. Multi-spectral images should be corrected for chromatic shifts. We have developed an efficient algorithm to address this problem (Section 2.1). There is also z-scaling present in practically all 3D images caused by refractive index mismatch [18]. In general, it is not possible to correct this distortion without additional information. We have proposed a solution in micro-axial tomography based on precise point-based image registration, where images from different views are available (Section 3.1).

Segmentation In the area of image segmentation, the most popular methods in the field are thresholding [65, 73], watershed [86], mathematical morphology operators [71, 79], variational approaches [59], level-sets [49, 72, 64], Markov random fields [24], and graph cuts [75, 5]. The selection of the right approach very depends on the application problem and objects of interest. Typical problems are 2D or 3D cell nucleus segmentation approached by graph-cuts [15], k-means and level-sets [12], watershed with model-based merging [46, 45], level-sets [88], deformable templates [23], or multi-scale techniques [29]. We have proposed

[11] Among the most popular publicly available software packages among biologists are ImageJ (http://rsbweb.nih.gov/ij/) [1] and CellProfiler [8, 44].

a method for 2D cell nucleus segmentation suitable for siRNA screening[12], which is superior to other commonly used methods in high-content screening especially for clustered nuclei (Section 4.1).

Another common segmentation problem is 3D spot detection [77]. This is very common in FISH studies where colocalizations of two targets are sought, because they indicate genetic translocations [68, 87]. For the spot detection, we have developed an approach based on HMAX-transform from mathematical morphology [79] which yielded very stable and reproducible results for the detection of endoplasmic reticulum exit sites in massive 3D confocal data sets (Section 2.4).

Classification Classifications in fluorescence microscopy are mostly related to machine learning approaches whose ultimate goal is to fully automatically learn different localization patterns of different proteins and to relate the localization patterns to protein function [10]. In general, classifications are often used to distinguish different cell phenotypes[13] [61, 32, 26, 11, 67]. We have developed an approach for cell classification as infected and non-infected by maximizing the difference between positive and negative controls (Section 4.1) and a measure for distinguishing compact and diffuse phenotypes (assembled and disassembled Golgi complex, Section 4.2) based on mathematical morphology operations.

Registration Image registration is similarly as image segmentation very broad research area [34, 92]. We have developed a rigid point-based registration approach for the elimination of the global movement of a living cell (Section 2.3). We have applied a similar approach to the alignment of micro-axial tomography data (Section 3.1). The main difference from the methodological point of view between these two approaches lied in the point pattern matching phase and the class of considered transformations. Whereas the class of transformations was a composition of 3D rotation and 3D translation in the former case, we have additionally searched for z-scale factor in the latter case. The point pattern matching problem was solved by a 3D generalization of an invariant[14] point pattern matching problem in the former case and by a reduction of the matching problem to an optimal assignment problem in bipartite graphs in the latter case. Note that elastic image registration has also been applied in fluorescence microscopy [17, 36, 16, 89, 20].

Tracking Tracking is usually necessary when dealing with live specimens [9, 21, 57]. In general probabilistic approaches overcome deterministic approaches in particle tracking [27]. Parametric active contours [91] and level-set-based tracking [19] were used to track the whole cells. We have developed a tracking approach considering appearance, disappearance, splitting, and merging of objects based on bipartite graph matching and maximum flows [42] (Section 2.3). In [48, 54], we have tracked the cells by a method based on agglomerative clustering (Section 4.2).

[12] siRNAs are short double-stranded RNA molecules which permit systematic knockout of related genes leading to silencing of the related protein. Each gene is typically attacked by several (often 3) different siRNAs. siRNA screening is common reverse engineering approach to discover gene/protein function [7, 14]

[13] cell phenotype = physical characteristics of the cell = how it can be seen

[14] invariant to rotation, translation, scaling and missing or extra points

1.3 Goal and Overview

The goal of the thesis is to concisely show my main results in the area of automated image analysis in fluorescence microscopy. The most natural way how to classify the results is to use the type of microscopy as the main criterion, because it highly correlates (1) to the main projects I have been working on, (2) to the type of image data, and (3) to the key image analysis problems I tackled. The main results are summarized in the next three chapters. Note that related work and relation to the big picture was shown in the previous section.

Chapter 2: Confocal Microscopy
Input images: Mostly multi-spectral 3D images
Main problems: image registration, tracking of sub-cellular structures, point pattern matching, 3D small object segmentation and quantification

Chapter 3: Micro-Axial Tomography
Input images: Tilted 3D gray-scale images
Main problems: image registration, point pattern matching, image fusion, resolution improvement

Chapter 4: Image Analysis in siRNA Screening
Input images: Many 2D multi-spectral images
Main problems: cell nucleus segmentation, whole cell segmentation, quantification, classification, tracking of cell nuclei, quality control

Chapter 2

Confocal Microscopy

In the area of confocal microscopy I have been working on several problems.

2.1 Chromatic Aberration Correction

Together with Michal Kozubek we have developed a method for the correction of chromatic aberrations [41]. The method is quick, precise, corrects lateral as well as axial aberrations, and is suitable especially for fluorescence microscopy where a limited number of fluorochromes are observed. It is based on 3D localization of fluorescent beads in 3D images and approximation of chromatic shifts by first and second order polynomials. The method has been in use in our laboratory since its invention and correction surfaces have been regularly computed and stored into a database for all microscopes maintained by our laboratory.

[41] M. Kozubek and **Pe. Matula**. An efficient algorithm for measurement and correction of chromatic aberrations in fluorescence microscopy. *Journal of Microscopy*, 200(3):206–217, 2000.

My contribution (**30%**): Design, development, and evaluation of the correction phase of the algorithm. Preparation of figures 3-7, collaboration on text writing. (fulltext on page **??**)

2.2 Automation of Confocal Microscopy

We have developed an automated system which combines confocal and wide-field acquisition modes to provide high-resolution and high-throughput measurements at the same time on FISH stained cells [39]. The system was an extension of a previous high-resolution cytometry instrument primary developed by Michal Kozubek [40] and it was called HRCM-2 (High-Resolution CytoMetry instrument). Later, we were developing a client/server-based solution for image cytometry [50]. During the development first versions of two open source

libraries (i3dlib[1] and v3dlib[2]) were created. The former, i3dlib, is a C++ template library for image representation and processing. The latter, v3dlib, is a C++ template library for orthogonal visualization of 3D images. Both libraries are used in image acquisition and image analysis software called Acquiarium[3] [51], which has been developed in our laboratory as a software solution for high-resolution cytometry.

[39] M. Kozubek, S. Kozubek, E. Lukášová, E. Bártová, M. Skalníková, Pa. Matula, **Pe. Matula**, P. Jirsová, A. Cafourková, and I. Koutná. Combined confocal and wide-field high-resolution cytometry of fluorescent in situ hybridization-stained cells. *Cytometry*, 45(1):1–12, 2001.

My contribution (**10%**): Design, development, evaluation, and testing of several parts of the system (most notably chromatic aberration correction module, but also collaboration on, e.g., switching between confocal and wide-field modes or manual segmentation of nuclei). (fulltext on page **??**)

[50] Pa. Matula, **Pe. Matula**, M. Kozubek, and P. Mejzlík. High-Resolution Cytometry Network Project: Client/Server System for 3D Optical Microscope Data Storage and Analysis. In: *Proc. of 2nd International Symposium on 3D Data Processing, Visualization, and Transmission (3DPVI 2004)*, Thessaloniki, Greece, 580–583, 2004.

My contribution (**20%**): Collaboration on the design and development of the system. Collaboration on text writing. (fulltext on page **??**)

[51] Pa. Matula, M. Maška, O. Daněk, **Pe. Matula**, M. Kozubek. Acquiarium: Free Software for the Acquisition and Analysis of 3D Images of Cells in Fluorescence Microscopy. In: *Proc. of IEEE International Symposium on Biomedical Imaging—From Nano to Macro (ISBI'09)*, 1138–1141, 2009.

My contribution (**10%**): A member of the team involved in the design of the initial versions of Acquiarium and an author of some of its parts related to data visualization (v3dlib). Collaboration on text writing. (fulltext on page **??**)

2.3 Image Registration and Tracking

We have extended the HRCM-2 system to permit high-resolution live cell imaging [42]. As the main contributions we have described a novel image acquisition strategies suitable for live cell studies and a novel tracking strategy based on graph theory to track sub-cellular objects. This tracking strategy was used in a real biological problem to track and analyze motion of HP1 foci [62].

We have developed a fast point-based registration method for the suppression of the global cellular movement [55]. It is based on extraction of significant intracellular objects and solving a point pattern matching problem and orthogonal Procrustes problem to find

[1] `http://cbia.fi.muni.cz/user_dirs/i3dlib_doc/i3dcore/index.html`
[2] `http://cbia.fi.muni.cz/projects/viewing-3d-images-with-v3dlib.html`
[3] `http://cbia.fi.muni.cz/projects/acquiarium.html`

the optimal composition of 3D translation and rotation in least-squares manner. The robustness of the method was evaluated on generated data and two real applications were presented.

[42] M. Kozubek, **Pe. Matula**, Pa. Matula, and S. Kozubek. Automated acquisition and processing of multidimensional image data in confocal in vivo microscopy. *Microscopy Research and Technique*, 64(2):164–175, 2004.

My contribution (**30%**): Design, development, and evaluation of a fast image registration and object tracking method based on graph theory. The algorithm developed in our previous paper [52] has been generalized to object tracking in case of appearance, disappearance, splitting and merging events. Collaboration on writing the paper. (fulltext on page **??**)

[62] V. Ondřej, S. Kozubek, E. Lukášová, M. Falk, Pa. Matula, **Pe. Matula**, and M. Kozubek. Directional motion of foreign plasmid DNA to nuclear HP1 foci. *Chromosome Research*, 14(5):515–514, 2006.

My contribution (**10%**): The algorithm developed in [42] has been applied to solve a real biological problem. The images were processed and analyzed in a close collaboration with the biologists. Collaboration on writing the paper. (fulltext on page **??**)

[55] **Pe. Matula**, Pa. Matula, M. Kozubek, and V. Dvořák. Fast point-based 3-D alignment of live cells. *IEEE Transactions on Image Processing*, 15(8):2388–2396, 2006.

My contribution (**70%**): Design and development of the fast point-based registration algorithm for eliminating global movement in 3D confocal microscopy images. Collaboration on evaluations and comparisons to alternative approaches. Writing the paper. (fulltext on page **??**)

2.4 Spot Segmentation and Quantification

We have worked on segmentation of small sub-cellular objects and estimating their spatial distribution [76]. The main objective was to decide if the objects are uniformly distributed inside cell nucleus or have significantly higher or lower density in the center. This was achieved by an approach based on top-hat filtering and computer simulations.

In collaboration with EMBL Heidelberg, we have worked on the quantification of the number of endoplasmic reticulum exit sites (ERES) in time series of 3D confocal fluorescence images [56]. The approach comprises: (1) Semi-automatic tracking of cells in maximum intensity projections, (2) 3D cell segmentation, (3) Segmentation of ERES (small dot-like objects), and (4) quantification of their number over time with respect to different phases of cell cycle. A novel method for 3D detection of small dots based on mathematical morphology has been developed.

[76] M. Skalníková, E. Bártová, V. Ulman, **Pe. Matula**, D. Svoboda, A. Harničarová, M. Kozubek, and S. Kozubek. Distinct patterns of histone methylation and acety-

lation in human interphase nuclei. *Physiological Research*, 56(6):797–806, 2007.

My contribution (**10%**): Collaboration on image analysis and simulation parts of the work and collaboration on writing its relevant parts. (fulltext on page **??**)

[56] Pe. Matula, F. Verissimo, S. Wörz, R. Eils, R. Pepperkok, and K. Rohr. Quantification of Fluorescent Spots in Time Series of 3-D Confocal Microscopy Images of Endoplasmic Reticulum Exit Sites Based on the HMAX Transform. In: *Proc. of the Conference on Medical Imaging 2010—Biomedical Applications in Molecular, Structural, and Functional Imaging, Proceedings of SPIE*, San Diego, USA, 7626:7 pages, 2010.

My contribution (**70%**): Design and development of the whole image analysis approach and its evaluation. Writing the paper. (fulltext on page **??**)

Chapter 3

Micro-Axial Tomography

One of the major limitations of most optical systems for fluorescence imaging is the spatial anisotropy of resolution[1]. To overcome this limitation a resolution improvement technique called micro-axial tomography based on tilting devices has been proposed [6]. The resolution improvement is achieved by acquisition of a set of tilted views of the sample, alignment of the tilted views, and fusion of the aligned images.

3.1 Automation and Resolution Improvement

We have worked on the automation of micro-axial tomography [43]. A strategy for automated acquisition and image processing has been developed. As an important part of the effort, we have developed (1) a point-based registration method to precisely align tilted 3D images of observed objects and (2) two image fusion procedures based on fusion either in spatial or frequency domain, which lead to resolution improvement [52]. The main advantages of the proposed method are high speed and high precision and accuracy. The precise registration method has been used to calculate real angular steps of the rotation motor, to correct z-scale distortion of images caused by refractive index mismatch [52] and to improve distance measurements between objects [80].

[43] M. Kozubek, M. Skalníková, **Pe. Matula**, E. Bártová, J. Rauch, F. Neuhaus, H. Eipel, and M. Hausmann. Automated micro-axial tomography of cell nuclei after specific labelling by fluorescence in situ hybridisation. *Micron*, 33, 7-8:655–665, 2002.

My contribution (**10%**): Collaboration on the automation of micro-axial tomography, in particular design and development of image registration and image fusion modules. (fulltext on page **??**)

[52] **Pe. Matula**, M. Kozubek, F. Staier, and M. Hausmann. Precise 3D image alignment in micro-axial tomography. *Journal of Microscopy*, 209:126–142, 2003.

[1] i.e., different resolution in all spatial directions, which is caused by diffraction and which is equal for confocal microscopy to about 0.25 μm in the lateral direction (in focal plane) and about 0.6 μm in the axial direction (along optical axis).

My contribution (**70%**): Design, development, and evaluation of a two-phase point-based registration method, in which point matching problem is reduced to an optimal assignment problem in weighted bipartite graphs. Writing the paper. (fulltext on page **??**)

[80] F. Staier, H. Eipel, **Pe. Matula**, A. V. Evsikov, M. Kozubek, C. Cremer, and M. Hausmann. Micro-axial tomography: A miniaturized, versatile stage device to overcome resolution anisotropy in fluorescence light microscopy, *Review of Scientific Instruments*, 82(9):8 pages, 2011.

My contribution (**10%**): Alignment of the images used in the work. Discussions on distance measurements. Revisions of the text. (fulltext on page **??**)

Chapter 4

Image Analysis in siRNA Screening

I have been working on several projects related to siRNA screening in the Biomedical Computer Vision Group, which is a part of the University of Heidelberg and German Cancer Research Center (DKFZ), Heidelberg.

4.1 Quantification of Viral Infection

We have developed a single-cell-based image analysis approach for the quantification of viral infection using cell arrays [22] and high-throughput microscopy [53]. A central issue is efficient, robust, and automated single-cell-based analysis of massive image datasets. For the segmentation of cell nuclei we have developed a novel, gradient-based thresholding scheme combined with mathematical morphology operations which does not require subsequent post-processing steps for separation of clustered nuclei and which yielded better results than other commonly used approaches[1]. The approach has been used in screening for human kinases[2], which are involved in virus entry and replication of hepatitis C and dengue viruses. One kinase screen based on cell arrays comprises around 20,000 images. Up today, we have analyzed around 10^6 images in several different projects. Because it is not possible to see all images we have developed also methods to check image quality (e.g., detection of out-of-focus images) [53].

A very important feature of the developed system is that it works on the single cell level and therefore spatial relations between cells can be studied [81]. This is important because it is known that virus infectivity depends on the density of cells [78]. The spatial localization of segmented cells can also be used for improved signal normalization [38].

The statistical analysis based on the single-cell-based normalization [38] was used to detect 13 different kinases involved in hepatitis C virus entry and replication [69]. The paper [69] is one of the main results of the VIROQUANT project I worked on in Ger-

[1] Comparison results obtained based on the same data

[2] Enzymes involved in energy transfer in cells coded by 719 genes, which were attacked by 2157 siRNAs (3 siRNAs per gene).

many during my post-doc stay. It is included to present importance of our image analysis approach and in particular importance of interdisciplinary research. Expertise and collaboration of many different people was necessary to obtain this highly cited paper[3].

[53] **Pe. Matula**, A. Kumar, I. Wörz, H. Erfle, R. Bartenschlager, R. Eils, and K. Rohr. Single-Cell-Based Image Analysis of High-Throughput Cell Array Screens for Quantification of Viral Infection. *Cytometry Part A*, 75A(4):309–318, 2009.

My contribution (**70%**): Design and development of the whole image analysis approach, its evaluation and comparison to other approaches. Writing the paper. (fulltext on page **??**)

[81] A. Suratanee, I. Rebhan, **Pe. Matula**, A. Kumar, L. Kaderali, K. Rohr, R. Bartenschlager, R. Eils, and R. König. Detecting host factors involved in virus infection by observing the clustering of infected cells in siRNA screening images. *Bioinformatics*, 26(18):i653–i658, 2010.

My contribution (**10%**): Complete image analysis of hepatitis C and dengue virus data. Help with writing image analysis parts of the paper. (fulltext on page **??**)

[69] S. Reiss, I. Rebhan, P. Backes, I. Romero-Brey, H. Erfle, **Pe. Matula**, L. Kaderali, M. Poenisch, H. Blankenburg, M. S. Hiet, T. Longerich, S. Diehl, F. Ramirez, T. Balla, K. Rohr, A. Kaul, S. Buhler, R. Pepperkok, T. Lengauer, M. Albrecht, R. Eils, P. Schirmacher, V. Lohmann, and R. Bartenschlager. Recruitment and Activation of a Lipid Kinase by Hepatitis C Virus NS5A Is Essential for Integrity of the Membranous Replication Compartment. *Cell Host & Microbe*, 9(1):32–45, 2011.

My contribution (**3%**): Single-cell-based image analysis of approximately 40,000 images (two full kinase screens) and quantification of fluorescence signal in cells infected by hepatitis C virus. Image analysis approach described in [53] was primary developed to detect interesting kinases for further investigation in this work. (fulltext on page **??**)

[38] B. Knapp, I. Rebhan, A. Kumar, **Pe. Matula**; N. A. Kiani, M. Binder, H. Erfle, K. Rohr, R. Eils, R. Bartenschlager, and L. Kaderali. Normalizing for individual cell population context in the analysis of high-content cellular screens. *BMC Bioinformatics*, 12:14 pages, 2011.

My contribution (**10%**): Image analysis of hepatitis C and dengue virus data. Writing image analysis related sections. (fulltext on page **??**)

4.2 Golgi Assembly and Disassembly

We have developed a live cell assay to study trafficking between endoplasmic reticulum and Golgi complex [48]. The key part of the live cell assay is its image analysis part [54]. Image

[3] The paper has been cited 17 times in 14 months.

analysis comprises: (1) nucleus detection, (2) nucleus tracking, and (3) quantification of Golgi complex localization patterns for single cell nuclei over time. We developed a new measure based on mathematical morphology for the quantification of the level of assembly or disassembly of the Golgi complex. It turned out that the new measure is significantly better than a previous frequently used measure to distinguish compact and diffuse phenotypes.

[48] T. Lisauskas, **Pe. Matula**, C. Claas, S. Reusing, S. Wiemann, H. Erfle, L. Lehmann, P. Fischer, R. Eils, K. Rohr, B. Storrie, and V. Starkuviene. Live-Cell Assays to Identify Regulators of ER-to-Golgi Trafficking. *Traffic*, 13(3):416–432, 2012.

My contribution (**20%**): Development of the image analysis approach used in the described live-cell assay. Image analysis of all data. Writing image analysis related sections. (fulltext on page **??**)

[54] **Pe. Matula**, T. Lisauskas, V. Starkuviene, and K. Rohr. Quantification of Golgi Complex Assembly and Disassembly in Live Cell Fluorescence Microscopy Images. In *Proceedings of Workshop on Microscopic Image Analysis with Applications in Biology*. Heidelberg, Germany, 4 pages, 2011.

My contribution (**70%**): Design and development of the whole image analysis approach and its evaluation. Writing the paper. (fulltext on page **??**)

Chapter 5

Conclusions

In this habilitation thesis I have presented a commentary to my work related to image analysis in fluorescence microscopy. The main contributions to the field are novel methods/algorithms for (1) point-based image registration of 3D confocal images and axial tomography data [55, 52], (2) chromatic aberration correction [41], (3) object tracking with split and merge events [43], (4) cell nucleus segmentation based on a combination of a gradient thresholding scheme and mathematical morphology operators suitable for high-content screening [53], (5) quantification of the level of assembly or disassembly of cellular phenotypes [54, 48], and (6) spot detection in 3D confocal data based on extrema dynamics measurements [56]. All proposed methods have successfully been applied for solving real biological or technical problems. This is presented by including relevant papers, which demonstrate practical usability of the developed methods [39, 43, 62, 76, 81, 38, 69, 80, 48].

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Part II

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