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Simulation framework of fluorescence microscopy movies to evaluate single cell quantification tools

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16.06.2014 _____

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Abstract

Single-cell analysis based on time-lapse microscopy allows monitoring the behavior of cells over time. Due to technical improvements in the last years, more and more time-lapse movies are available. At the same time, the number of image processing tools, which analyze microscopy data automatically increases.

Quantification of tracked fluorescence (QTFy) is such a tool and measures cellular fluorescence signals of single-cells in time-lapse microscopy movies. Previously to the intensity quantification, the tool estimates the background of each image of a movie and performs a normalization. To evaluate the error in the quantification of single cell fluorescence of QTFy and similar tools, curated test data is necessary. Due to a lack of well-annotated image data, the use of synthetically created cell images is necessary.

Here, an existing microscopy image simulating tool was extended so that it is able to generate simulated time-lapse microscopy movies. Based on that, a pipeline was implemented, which generates movies with specific cell and image properties. On the generated movies, the background estimation and fluorescence quantification of QTFy was applied. Finally, the signal quantification as well as the quality of the estimated backgrounds are assessed within the pipeline. This approach was applied to test scenarios with a variety of parameters and the generated data was analyzed. Thereby an examination of the influence of different movie and cell properties on the quantification performance of QTFy was carried out. The results of this analyzes show that the largest quantification error of the investigated tool emerge from errors in the estimation of the background. We assigned our analysis results to real fluorescence microscopy movies of differentiating blood stem cells and pluripotent embryonic stem cells and estimated an average relative deviation of the measured cell intensity of about 5% due to quantification errors.

The implemented pipeline can serve as basis for further evaluations of QTFy to account for further effects like photo bleaching, specific cell shapes or cellular movements.

Zusammenfassung

Einzelzellanalyse mittels zeitaufgelöster Mikroskopie ermöglicht das Verhalten von Zellen zu beobachten. Aufgrund der technischen Entwicklungen der letzten Jahre gibt es immer mehr solcher Zeitrafferfilme und dementsprechend immer mehr Bildverarbeitungsprogramme, die die Mikroskopiedaten automatisiert analysieren.

QTFy (Quantification of tracked fluorescence) ist ein Programm das Fluoreszenzsignale auf Einzelzellebene in Zeitrafferfilmen misst. Bevor die Fluoreszenzintensitäten gemessen werden, nimmt das Programm für jedes Bild des Filmes eine Schätzung des Hintergrundes vor. Darauf basierend werden die Bilder normalisiert. Um die Quantifizierung von Einzelzellen durch QTFy und verwandte Programme zu evaluieren sind adäquate Testdaten nötig. Da es zu wenige gut annotierte Mikroskopiedaten gibt, bietet es sich an, simulierte Zellbilder für die Beurteilung zu verwenden.

Im Rahmen der vorliegenden Arbeit wurde ein existierendes Programm, das Mikrospiebilder simuliert, so erweitert, dass es simulierte Zeitrafferfilme generiert. Darauf basierend wurde eine Pipeline implementiert, die Filme mit bestimmten Zell- und Bildeigenschaften generiert. Auf die so generierten Filme wurde die Hintergrundschätzung sowie die Fluoreszenzsignalmessung von QTFy angewendet. In der Pipeline werden dann die Signalmessungen sowie die Qualität der geschätzten Hintergrundbilder bewertet. Die Pipeline wurde auf verschiedene Testszenarien angewendet und die gewonnen Daten analysiert. So wurde der Einfluss von verschiedenen Bild- und Zelleigenschaften auf die Signalmessung untersucht. Die Ergebnisse dieser Analysen zeigen, dass die größten Fehler des untersuchten Programms bei der Fluoreszenzmessung meist auf Fehlern bei der Schätzung des Hintergrundbildes basieren. Wir ordneten reale Filme differenzierender Blutstammzellen und pluripotenter embryonaler Stammzellen unseren Analyseergebnissen zu und schätzten die durchschnittliche Abweichung der gemessenen Zellintensitäten aufgrund von Quantifizierungsfehlern auf etwa 5%.

Die implementierte Pipeline kann als Grundlage für weitere Validierungen von QTFy dienen. Durch Erweiterungen könnten Filme simuliert werden, die zusätzliche Effekte wie Photobleichung, bestimmte Zellformen und Zellbewegungsmuster mit einbeziehen.

Contents

1 Introduction

1.1 Biological Background

1.1.1 Single cell

The research field of cell populations helps to get a deeper understanding of diseases and variations between humans. For a long time cells within the same cell population were expected to be equal. In contrast to the earlier assumption cells within one population are not homogenous (Nobs & Maerkl, 2014; Spiller, Wood, Rand, & White, 2010). There are differences in the characteristics of cells within one population. Methods which focus on the averages of heterogeneous populations ignore the properties of single cells (for a comment see Schroeder, 2011). To understand the plasticity of cells and the regulation of fate decisions, like cell division, differentiation and apoptosis and answer long-standing biological issues however it is necessary to observe single cells continuously (Schroeder, 2011; Spiller et al., 2010). With this approach, several insights have already been gained, as for example by the observation of mouse mesodermal cells generating endothelial cell and blood colonies (Eilken, Nishikawa, & Schroeder, 2009) or by studying the behavior of human glioblastoma cells in vitro (Demuth et al., 2000). Figure 1 shows that an average cell content can be distributed to the singles cells in different ways.

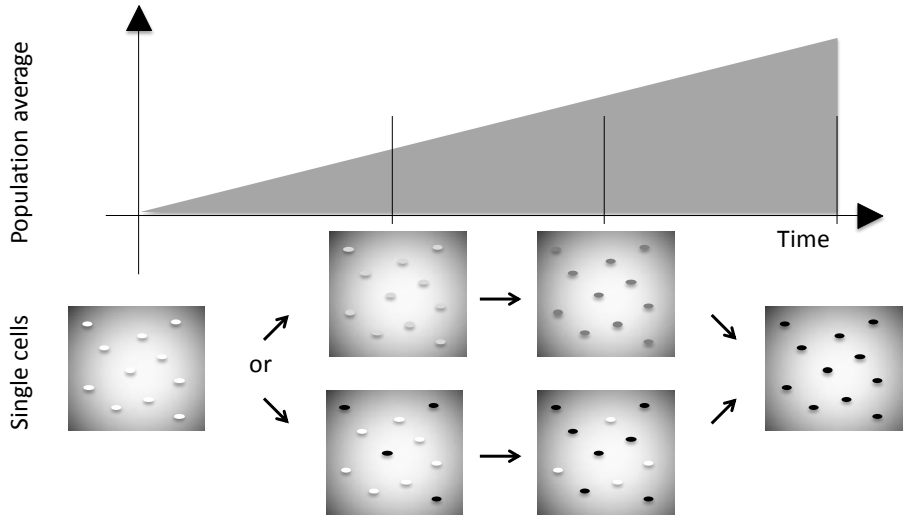

Figure 1 Heterogeneity of single cells is not detectable in population average studies. The content of a specific protein increases over time in a cell population (top). But what that means for single cells is obscured. There are different possibilities how the increasing protein content could be distributed on the single cell. One option is that the protein content increases in all cells simultaneous (gray ovals), but it is also possible that there are cells with highly upregulated protein (black ovals) and cells, that do not contain the specific protein (white ovals) within the population.

Snapshot analyses rely on cell measurements of a single time point. Such analyses of cells during differentiation do only reveal the result of a number of fate decisions and obscure the single steps which have led to this results over a unknown number of generations (Rieger & Schroeder, 2008). Figure 2 shows potential scenarios, which could have led to the final proportion of heterogeneous cells. In each scenario the resulting amount of cells are two cells with slightly upregulated protein (shown as gray ovals) and two cells with strongly upregulated protein (shown as black ovals), but the sequence of fate decisions of the individual cells (white ovals) differs in each picture and can only be determined by continuous single-cell observation (Coutu & Schroeder, 2013).

Since the importance of single cell analysis has been noticed and the image technology improved more and more continuous live-cell long-term studies are performed (Demuth et al., 2000; Eilken et al., 2009; Nobs & Maerkl, 2014). It is very difficult to observe cells in living organisms, particularly in mammals, over more than a few hours. Therefore in vitro imaging methods, where cells are studied in a culture medium instead of their normal biological environment are practicable alternatives where cells can be observed for up to several weeks (Schroeder, 2011).

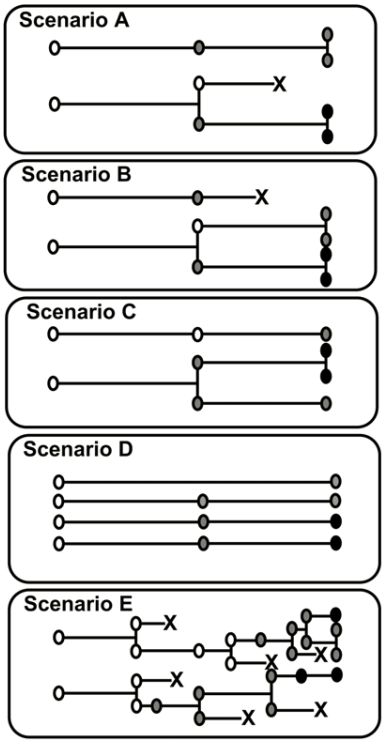

Figure 2 Snapshot analyses conceal the dynamic processes that lead to specific cell characteristics. Each of the five different scenarios (A-E) results in two cells with strongly upregulated protein (depicted as black ovals) and two cells with mildly upregulated protein (gray ovals). However, the steps leading to this result differ in each scenario and can only be discovered by continuous single-cell imaging Figure adapted from (Coutu & Schroeder, 2013).

1.1.2 Cell fluorescence

To analyze living cells by time-lapse microscopy, cells have to be visualized non-invasively. One technique to do that, is to tag proteins with fluorescent dyes (Lippincott-Schwartz & Patterson, 2003). The green fluorescent protein (GFP) was first isolated from the jellyfish Aequorea victoria in 1962 (Shimomura, Johnson, & Saiga, 1962). In living organisms this fluorescent protein can be used to monitor gene expression. Therefore the promoter of a gene of interest has to be attached to the coding region of the GFP gene. In this way the simultaneous expression of GFP and the gene of interest can be observed and the fluorescent signal can be used to quantify the expression (Andersen et al., 1998; Miyashiro & Goulian, 2007). Today there are several variants of GFP which have been modified and can exhibit fluorescence in different color. Thus proteins with distinct spectral properties can be attached to the different cell compartments and the subcellular location of the protein can be visualized under the microscope (Coutu & Schroeder, 2013).

Fundamental problems at the visualization are phototoxicity and photobleaching. These are light induced damages of cell components and culture medium respectively. Excited fluorophores produce reactive oxygen species, which causes a reduction of fluorescence signal (photobleaching) and damages to the cell (phototoxicity) (Hoebe et al., 2007). Consequently, the more excitation-light is applied, the better is the visualization of fluorescence signals but the higher are the damages to cell components and the bleaching of the culture medium.

1.1.3 Single cell quantification

Before the fluorescence intensity and therefore the protein content of single cells can be quantified using fluorescence microscopy imaging techniques, further information may be used. First of all, the cells to be investigated have to be tracked if it is a time-dependent study so that the positions of the cells within the images are known. The next step on the way of cell quantification is the segmentation of each cell to be quantified, so that the exact boundary of the cell is known. Moreover the background has to be normalized, due to the aforementioned microscopy image characteristics, like illumination properties, noise and autofluorescent background. With this information the absolute intensity of a cell can be measured by summing up the intensity of each pixel within the cell boundaries (Schwarzfischer, 2013).

1.2 Technical Background

1.2.1 Time-lapse microscopy

Time-lapse microscopy is a technique which is known since the late 1890s and yielded the first scientific success in 1909, when Jean Comandon observed the movement of syphilis bacteria (Landecker, 2005). Applied to fluorescent microscopy it can be used to measure cell properties like the content of a specific protein and other cytometric properties (Miyashiro & Goulian, 2007; Muzzey & van Oudenaarden, 2009). To observe single cells and their fates over a long period a main challenge is to keep the cells alive and not change their original

properties (Coutu & Schroeder, 2013). At the same time images in a good quality and methods for their storage and analysis are indispensable.

1.2.2 Image Acquisition

The technical resources for time-lapse microscopy have improved over the last years, but there is not one single solution that works well for all experiments. The technical equipment has to be chosen and adjusted in accordance to the cells to be investigated in their culture medium.

Due to phototoxicity and photobleaching, for long-term imaging a tradeoff between good pictures and healthy cells is required (Schroeder, 2011). For low fluorescence signal objectives with a large aperture are recommended, whereas for high fluorescent signals objectives that can minimize exposure times reduce phototoxicity (Coutu & Schroeder, 2013).

The type of the used lamps will influence the study results. There are several options for the choice of illumination, such as metal halide lamps, mercury lamps, Xenon or LED lamps (Lichtman & Conchello, 2005) and the challenge is to take a light source that leads to images in good quality but minimize the phototoxicity of cells and culture medium. Currently mercury and metal-halide arc lamps are the light source of choice for fluorescence microscopy images (Coutu & Schroeder, 2013). But the progression in the light emitting diode (LED) technology makes advantageous lamps available, which are small, energy saving and provide adequate intensities at specific wavelength (Carl Zeiss MicroImaging, 2007). If compatibility problems to existing microscopy imaging systems will be resolved, the metal-halide arc lamps might be replaced by LED lamps in future (Coutu & Schroeder, 2013).

There are different types of suitable cameras. Until now in most cases charge-coupled device (CCD) cameras were chosen, due to their sensitivity and low noise. However, nowadays CCD cameras are often replaced by complementary metal oxide sensor (CMOS) cameras, because they outperform CCDs (Bioimager, n.d.; Faruqi, Henderson, Pryddetch, Allport, & Evans, 2005).

Beside the type of camera there are several experimental setup properties like exposure time, chip size, resolution, noise, and many others which have to be taken into account for time-lapse microscopy images (Coutu & Schroeder, 2013).

Despite the technical advancements of devices and refinements that can be achieved by excellent adaption to the individual needs, measurement noise in fluorescence microscopy images cannot be avoided completely (Coutu & Schroeder, 2013; Lehmussola, Ruusuvuori, Selinummi, Huttunen, & Yli-Harja, 2007). This noise as well as uneven illumination and autofluorescence of the culture medium are the problems with which the methods that analyze these images has to deal with (Lehmussola et al., 2007).

1.3 Existing software tools

1.3.1 Description of QTFy

Quantification of tracked fluorescence (QTFy) is a tool, written in MATLAB, for the semi-automated quantification of cellular fluorescence of tracked cells in time-lapse microscopy experiments (Schwarzfischer, 2013).

The first step of QTFy is the normalization of the images. With this process (see Chapter 2.3.3 for details) uneven illumination and background signal can be corrected, making the cell signals at different image positions comparable.

Prior to the quantification, the cells to be examined need to be tracked and segmented. The tracking can be executed by a tracking tool of choice, e.g. by TimeLapseAnalyzer (Huth et al., 2010, 2011), TLM-Tracker (Klein et al., 2012) or Celltracker (Scherf et al., 2012). The segmentation of the tracked cells is performed by QTFy automatically. The user can choose which channel(s) should be used for the segmentation. The background correction also improves the cell segmentation. To solve potential segmentation errors, the user has the possibility to inspect each cell at each time point and modify the segmentation parameters or even draw a cell shape by hand with the graphical user interface provided by QTFy.

In the next step some segmentation settings, like fluorescence channels, segmentation algorithm, threshold and the minimum and maximum of expected cell size need to be made. The channels for segmentation and quantification may be the same, but do not have to. When the selection of the basic settings is completed, the segmentation runs automatically and the quantification based on the segmentation is performed (Schwarzfischer, 2013). In Figure 3 the general workflow of QTFy is shown.

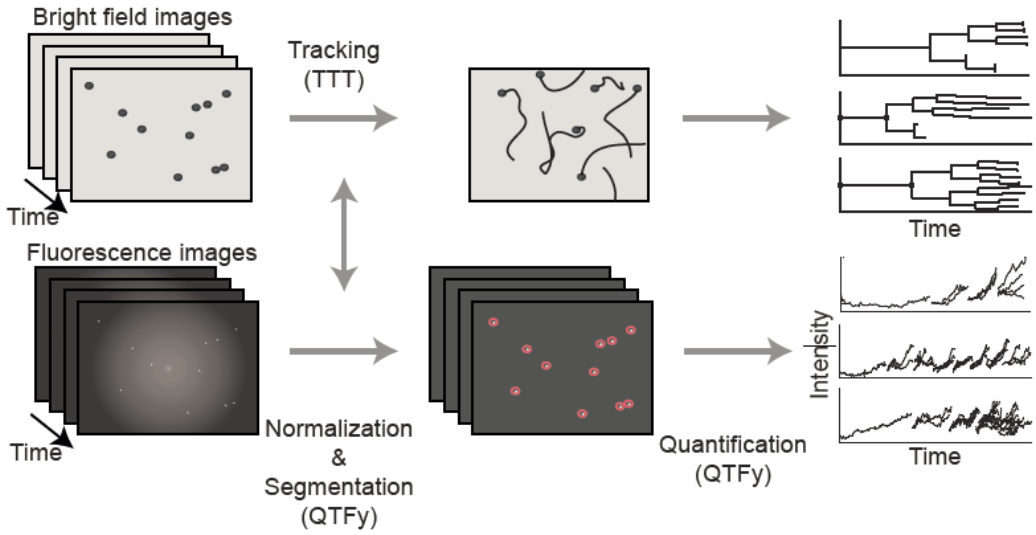

Figure 3 QTFy is a tool that quantifies fluorescence intensities of single cells in time-lapse microscopy movies. The first steps of QTFy are the normalization of the fluorescence time-lapse microscopy images (see Chapter 2.1.4 for a detailed description) and the segmentation of the cells. Following the cells are quantified. Connected to the tracking information, the fluorescence intensities are visualized over time (Schwarzfischer, 2013). Figure adapted from (Schwarzfischer, 2013)

1.3.2 Description of SIMCEP

Simulating Microscopy Images with Cell Populations (SIMCEP) is a tool for generating/simulating synthetic images of fluorescent cell populations. These images with realistic properties can be used to evaluate methods which analyze digital fluorescence microscopy images. The image simulator is written in MATLAB and freely available for scientific use (Lehmussola et al., 2007).

The tool has several variable parameters which can be controlled by the user and can be customized to different image properties/characteristics.

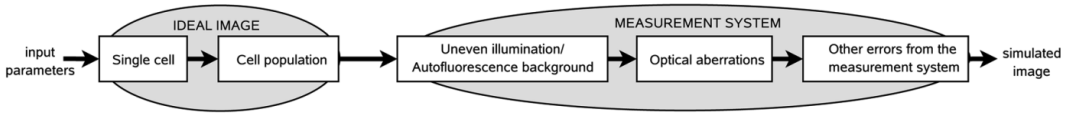

Figure 4 Pipeline of the proposed simulation process of SIMCEP. Using input parameters from the user, the generation of fluorescence microscopy images starts with the simulation of single cells and cell populations. This ideal image is then transformed to make it more realistic. Therefore some disturbances like uneven illumination and noise, simulating the image acquisition are added Figure taken from (Lehmussola et al., 2007).

The pipeline of the simulation process is as follows (compare Figure 4). First, an ideal image with the parameters specified by the user is generated. This image does not contain any errors from measurement and may serve as reference image.

The following parameters can be adjusted:

- The size of the picture
- The number of cells, which are randomly placed in the image
- The number of cell clusters in which the cells are placed
- The probability that a cell is assigned to a cluster
- Are cell overlaps allowed
- Should overlaps be measured on nuclei or cytoplasm level
- Adjustments for the appearance of the cells (size, visualized cell compartments, shape)

With the given probability each cell is assigned to a cluster and these are distributed uniformly over the image. Within the clusters the cells are randomly distributed around the cluster center. Cells which are not assigned to a cluster are randomly placed on the. In Figure 5, we show exemplary spatially dispersed (A) and clustered (B) cell nuclei.

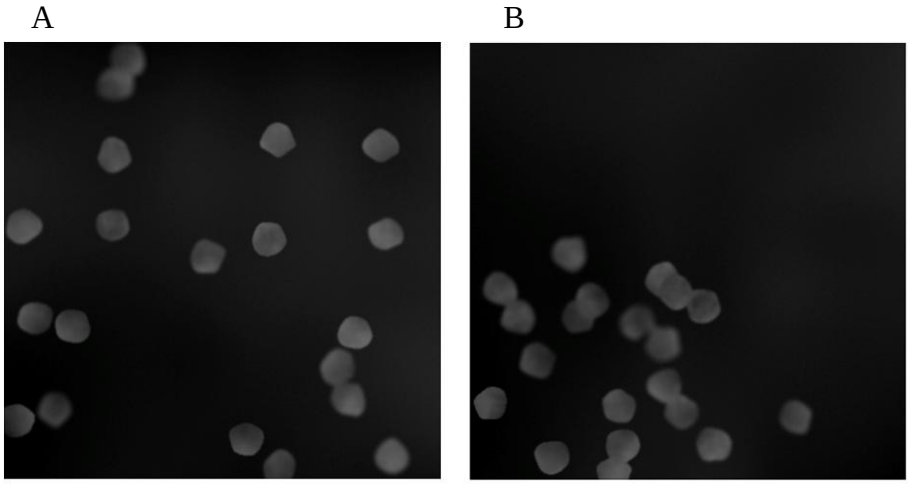

Figure 5 SIMCEP allows simulating fluorescence images with various appearances including cell clustering behavior. (A) The cell nuclei are spatially dispersed and randomly distributed over the image. (B) The nuclei are clustered together and randomly distributed within the cluster. Figure adapted from (Lehmussola, 2007).

The cells consist of the three components nucleus, cytoplasm and sub cellular objects. For simulated images a subset of these components can be selected. For each of these components the user can determine all parameters for the calculation of the cell, such as shape, radius and texture components. In addition the number of sub cellular components can be set.
The next step is to make the image more realistic by adding the following parameters: Background signal, due to the autofluorescent culture medium, uneven Illumination, optical deviations and noise, reflecting errors due to the measurement system.

The user can set the energy of illumination and energy of autofluorescence compared to the intensity of cells, variance of noise for the CCD detector and the amount of compression artifacts.

As the MATLAB source code for the tool is freely available and customizable, SIMCEP can be adaptable to our requirements. The generated synthetic images with known ground truth can be used for the validation of different analysis methods, as the analysis results can be compared with its ground truth.

1.4 Aim of this thesis

The technical improvements in microscopy imaging is accompanied by an increasing number of automated image cytometry tools. To assess such tools, curated test data is indispensable. Due to a lack of well-annotated natural image data, the use of synthetically created cell images allows a thorough evaluation of these tools.

The workflow for the evaluation of such tools is as follows. The first step is to generate images with known cell properties, like the coordinates of the cells, the cell size, the intensity of the cellular fluorescence, the amount of background signal, and the shape of the

illumination. These images are referred to as ground truth. In the next step the cytometry analysis tool, which shall be evaluated, gets these images as input and performs its calculations. Finally, the results can be compared to the ground truth, for example by calculating the RMSD (see Chapter 2.5.1). By this means we can assess the performance of an analysis tool.

For the evaluation of QTFy, we extended the image simulation tool SIMCEP, so that movies are generated instead of images and adapted the properties to our requirements. Using this customized version, we developed a pipeline for the evaluation of QTFy and analyzed the measured fluorescent signals on a single-cell basis to quantify the influence of cell intensity, illumination, noise and the signal to background ratio on the quantification performance of QTFy.

1.5 Overview of this thesis

The rest of the thesis is organized as follows. In Chapter 2 we introduce the methods, QTFy uses for the estimation of the background, the image normalization, the cell segmentation and the cell quantification. Then we explain the methodical background for the simulation of fluorescence microscopy images and describe the basic statistical concepts we applied to evaluate the analysis results. In Chapter 3 we present the single steps of the pipeline we implemented to evaluate the performance of QTFy and describe our test data. The results of these test cases are represented and discussed with regard to QTFy in Chapter 4. Chapter 5 summarizes all meaningful information of this thesis and gives an outlook about possible extensions of the evaluation pipeline.

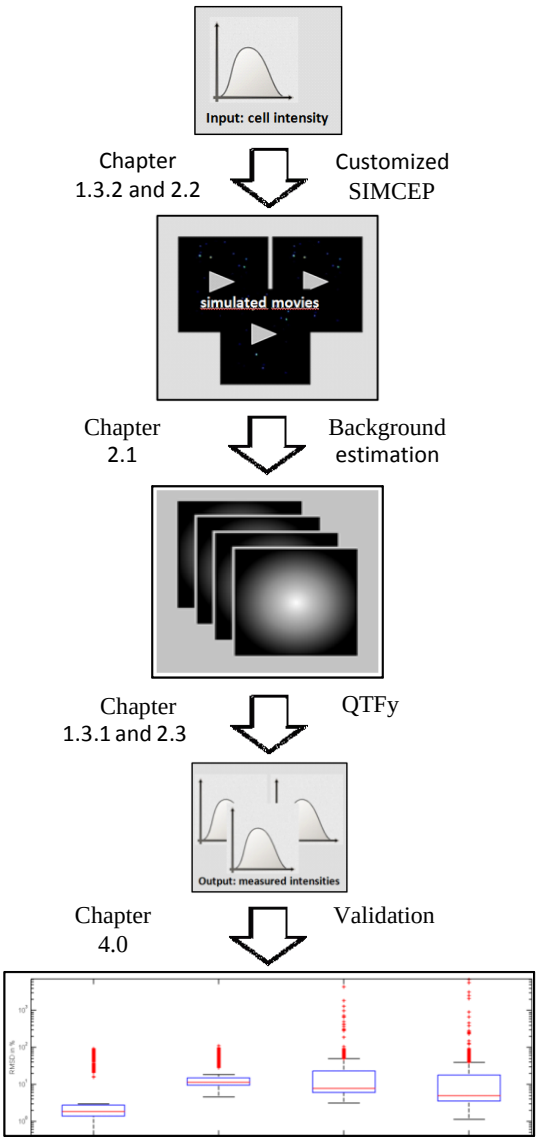

Figure 6 Thesis overview. We use a customized version of SIMCEP to simulate fluorescence microscopy images with known cell fluorescences (see Chapter 1.3.2 for a description and Chapter 2.2 for methodical information). The backgrounds of these simulated images are estimated by QTFy or median filtering (see Chapter 2.3). After these background were used to normalize the simulated images, QTFy measures the cell signals (see Chapter 1.3.1 and 2.4). To validate the analysis results we compare them to the simulated cell signals and analyzed the influence of different image properties on the quantification performance of QTFy (see Chapter 4).

2 Methodological background

In this section, we describe basic methods, which are used within our pipeline to simulate images, estimate the backgrounds of the simulated images, quantify cells and evaluate the output of the background estimation and cell fluorescence intensity quantification.

2.1 Image model and definitions

A typical fluorescence microscopy image is defined as $I(x;t)$, where x contains the space coordinates $x = (x1;x2)$ and t is the time point of the image. Such an image consists of the following components (illustrated in Figure 7):

- the time- and space-dependent cell signal $s(x;t)$
- the homogenous background signal $b(t)$, which comes from the autofluorescence of the culture medium
- the illumination function $g(x)$, which is pixel specific an represents the uneven illumination
- the camera offset $o(x)$
- Noise caused by the measurement system is assumed to be constant over time and position. Therefore noise can substantially eliminated by robust fitting methods used for the normalization and is not considered for the calculation of the background.

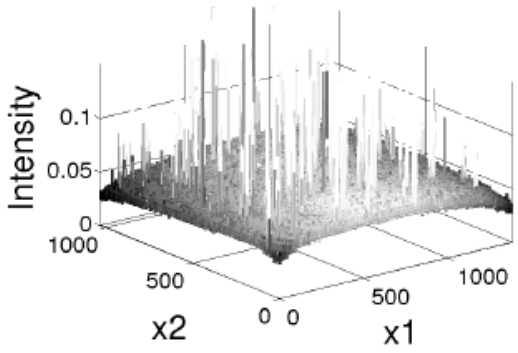

Figure 7 A fluorescence microscopy image from a time-lapse microscopy movie consist of varying fluorescence intensities (plotted on the z-axis) for each pair of coordinates $(\boldsymbol{x1}, \boldsymbol{x2})$. Intensity peaks represent cellular signal. Beside the cellular signal, the intensities in time-lapse microscopy images are influenced by an uneven illumination, a decreasing intensity of autofluorescence of the culture medium and the camera offset (Schwarzfischer et al., 2011) (Figure adapted from (Schwarzfischer et al., 2011).

The following equation represent how the single image components are related

$$I(x;t) = s(x;t) \cdot g(x) + b(t) \cdot g(x) + o(x) \quad (1)$$

Therefore the cell signal $s(x;t)$ can be expressed by

$$s(x;t) = \frac{I(x;t) - b(t) \cdot g(x) - o(x)}{g(x)} \quad (2)$$

due to photo-bleaching of the culture medium, the background signal $b(t)$ decreases over time.

To take this into account the illuminated background signal $B(x;t)$, defined as

$$B(x,t) = b(t) \cdot g(x) + o(x) \quad (3)$$

is calculated for each image of the time-lapse microscopy movie independently (see Chapter 2.3).

2.2 Simulation of fluorescence microscopy images with SIMCEP

To simulate fluorescence microscopy images, we use the tool SIMCEP (Lehmussola et al., 2007; Lehmussola, Ruusuvuori, Selinummi, Rajala, & Yli-Harja, 2008) (see Chapter 1.3.2). In the following we will discuss the properties and parameters of the simulation framework and their methodical application for the generation of synthetic microscopy images (Lehmussola et al., 2007, 2008)

2.2.1 Cells

The following equations describe the coordinates $(x(\theta), y(\theta))$ of a circle on which the random shape of a cell are based

$$x(\theta) = cos(\theta) \quad (4)$$

$$y(\theta) = sin(\theta) \quad (5)$$

with the polar angle $\theta \in [0, 2\pi]$. After creating a regular polygon with k vertices and sampling of angle θ with equal distance (see Figure 8 A), the coordinates of the vertices are randomized.

These randomized vertices are specified as

$$x_i(\theta_i) = r[U(-a;a) + cos(\theta_i + U(-b;b))] \quad (6)$$

$$y_i(\theta_i) = r[U(-a;a) + sin(\theta_i + U(-b;b))] \quad (7)$$

for $i = 1, \ldots, k$ where $U(a, b)$ is a uniform distribution on the interval $[a, b]$. The last step is to apply a cubic spline-interpolation (see Figure 8 B), which connects the vertices resulting in a smooth and flexible contour for the random shapes (see Figure 8 C and D). Highly distorted shape (for example see Figure 8 D) are more typical for cytoplasm. To simulate the nuclei in our movies, we used slightly irregular random shapes similar to Figure 8 C (see Chapter 3.1.1 and Figure 15).

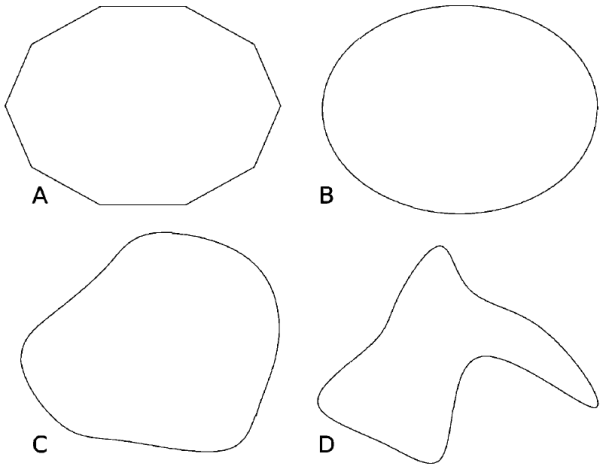

Figure 8 Generation of random shapes. (A) A regular polygon before the coordinates of the vertices are randomized. (B) Resulting shape when the vertices of (A) are connected with spline interpolation. (C) Example of a slightly irregular random shape. (D) Example of a more distorted shape (Lehmussola et al., 2007). Figure taken from (Lehmussola et al., 2007)

2.2.2 Illumination

The next step in simulating fluorescence microscopy images is to add confounding factors, which are caused by the measurement systems in the original microscopy image (see Figure 4). One of these factors is the illumination of the experiment. Due to technical limitations, this illumination is unevenly distributed. To simulate this, a second degree parabolic polynomial is added to the image with cells. Then a mesh grid is constructed, with the size of the image. In this grid the inner coordinates are filled with ones, which stands for white pixels. The farther one moves outside the smaller is the content of the coordinates. Around the edges the numbers go towards zero. This matrix is than scaled by the illumination factor, which controls the energy of illumination. The resulting matrix can be regarded as the position dependent background signal $B(x, t)$ (see Chapter 2.1,Equation (3) and Figure 9 B).

To adapt the generation of the illuminated image to the used normalization method (see Chapter 2.3.3 and Equation (12)), the background signal $B(x)$ is added to the image containing the simulated cells $s(x)$, in the following way. First the image with the cells $s(x)$ is multiplied with the background signal $B(x)$. Then the background signal $B(x)$ is added to the resulting matrix (see Figure 9 C).
As equation

$$I(x, t) = s(x, t) \cdot B(x, t) + B(x, t) \tag{8}$$

2.2.3 Noise

Light consist of single particles, which are called photons. These photons are counted by CCD detectors. The number of photons, collected by the detector varies and can be described with Poisson statistic (Meyer & Kirkland, 2000). This results in a Poisson distributed shot noise in images.

The Poisson distribution is defined as

$$P_n = \frac{\lambda^n \cdot e^{-\lambda}}{n!} \tag{9}$$

where e is Euler's number, $\lambda \in \mathbb{R}_{>0}$ is the mean, n is the number of photons and $n!$ is the factorial of the n (DeGroot & Schervish, 2012).

The extent of shot noise increases proportionally to increasing light intensity. For large numbers of events, which here means high intensities, the normal distribution (with standard deviation $\sqrt{n}$) is a good approximation to the Poisson distribution.

Therefore the signal to noise ratio (SNR) can be defined as

$$SNR = \frac{n}{\sqrt{n}} \tag{10}$$

where n is the number of expected events (Imaging Scientific Volume, 2014).

Since the noise increases according to the square root of light intensity, the relative proportion of noise decreases because the signal to noise ratio (see Equation (10)) increases with the intensity of light. The brighter an image is, the less it is influenced by shot noise.

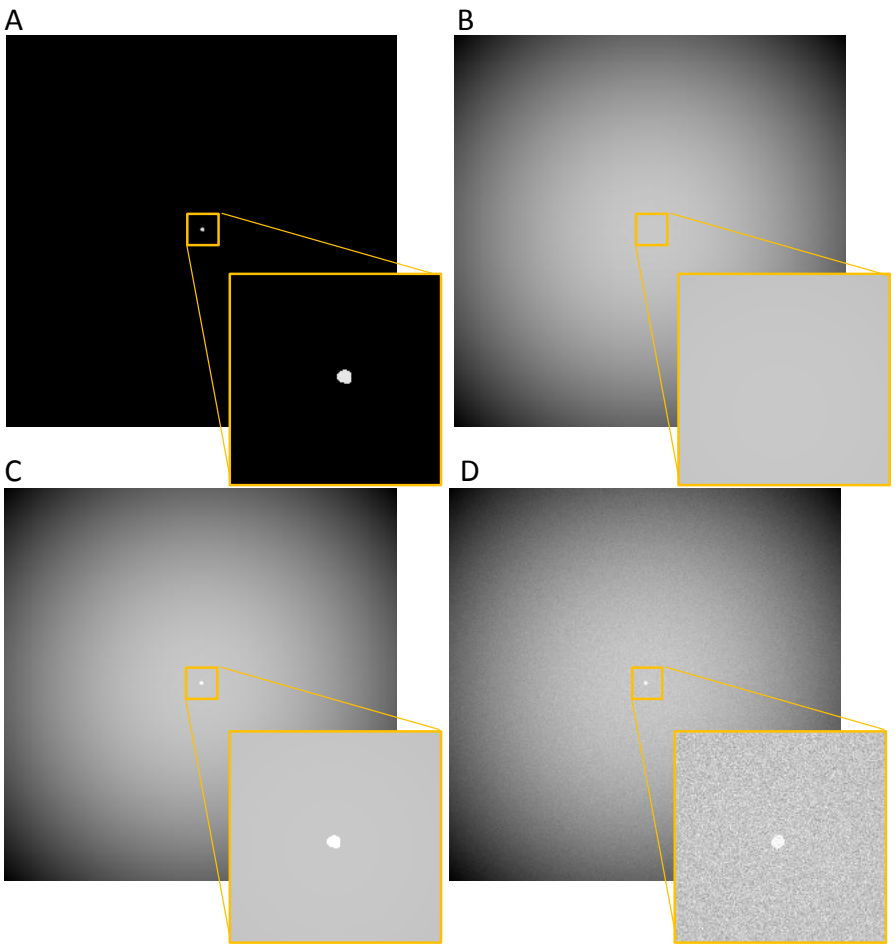

Figure 9 Components of simulated fluorescence microscopy images. (A) This image contains one simulated cell with a pixel intensity of 0.8 and no background signal. (B) Simulation of an uneven illuminated background $\boldsymbol{B}(\boldsymbol{x})$ (see Chapter 2.2.2). (C) Multiplicative and additive merge of the images (A) and (B) (see Chapter 2.2.2). (D) Poisson distributed shot noise was added to image (C) (see Chapter 2.2.3).

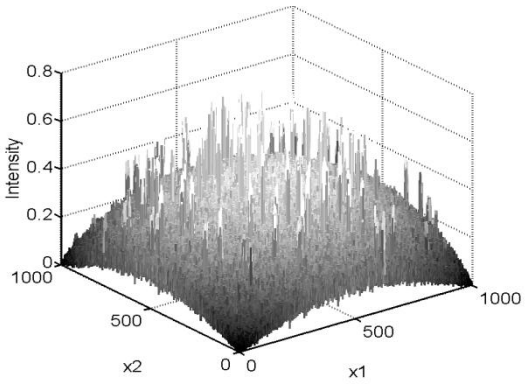

Figure 10 Exemplary simulated fluorescence microscopy image with space coordinates $(\boldsymbol{x1}, \boldsymbol{x2})$. The z-axis represent the intensity of each pixel. This image contains: (i) Inhomogeneous illumination $\boldsymbol{B}(\boldsymbol{x})$ (see Chapter 2.2.2), (ii) Poisson distributed noise (see Chapter 2.2.3) and (iii) 100 cells with a pixel intensity of 0.5 ($\boldsymbol{s}(\boldsymbol{x})$) (compare to Figure 7).

The MATLAB method imnoise(I, 'poisson') (Mathworks, 2014b) adds Poisson distributed noise to the image $I(x)$, which constists of the cell signal $s(x)$ and possibly of the illuminated background signal $B(x)$. The input pixels are interpreted as the means of Poisson distribution. Each space coordinate x of image I is replaced by sampling from a Poisson distribution with the given mean (see Equation (9)). For examples see Figure 9 D and Figure 10.

2.2.4 Random walk

To generate movies from the simulated images, we need to simulate the movement of a cell. We do that by calculating new space coordinates of a cell for every image in the movie. For this purpose, we use the mathematical random walk model, where the single steps occur randomly in space.

This stochastic process is characterized by independent and identically distributed increments and is defined as

$$X_a = X_0 + \sum_{j=1}^{a} Z_j \qquad (11)$$

where $Z_1, \ldots, Z_j$ is a sequence of independent random variables, with values from $\mathbb{R}^2$, $a \in \mathbb{N}_0$ and X_a is a 2-dimensional random walk (WolframMathworks, 2014).

2.3 Background estimation and image normalization

To be able to quantify fluorescence intensity of cells, their signals need to be made comparable. Due to uneven illumination and autofluorescence of the culture medium the background of fluorescence time-lapse microscopy images needs to be normalized to reach a comparable level. In the following, two methods for background estimation are described (Schwarzfischer et al., 2011).

2.3.1 Background estimation by median filtering

Median filtering is a nonlinear method, which is commonly used for the suppression of impulse noise. At this type of noise only a part of the pixels of an image is noisy and these pixels are either very bright or very dark (He et al., 2011).

In this filtering method, each pixel of an image is replaced by the median value of its neighboring pixels (Mathworks, 2014c; Steger, Ulrich, & Wiedemann, 2007). The area of considered neighbors is called window. The size and shape of the neighborhood depends on the application (Nixon & Aguado, 2012; Petrou & Petrou, 2010). In this thesis we used the median filtering to estimate the background of simulated fluorescence microscopy images and adapted the window therefore to the approximate cell size.

2.3.2 Background estimation by QTFy

The first step is the division of the image $I(x;t)$ into small sub-images, with overlapping edges (see Figure 10). Next, these so called tiles are grouped into tiles with cell signal and tiles without cell signal. The distinction can be made by the moments of the distribution of intensities, which differ substantially for the two types of tiles. Based on these statistical features and the density-based clustering method DBSCAN (Ester, Kriegel, Sander, & Xu, 1996), two clusters are formed: one cluster with high density, which contains only tiles without any cell signal and one heterogeneous cluster, which contains tiles with cellular signal (see Figure 10). From each background tile, stored in the first cluster, the median is used for the construction of a first background mesh. The last step for the estimation of the time-dependent background signal $B(x;t)$ is the application of a two dimensional natural neighbor inter- and extrapolation (see Figure 10). This process is applied to every image independently.

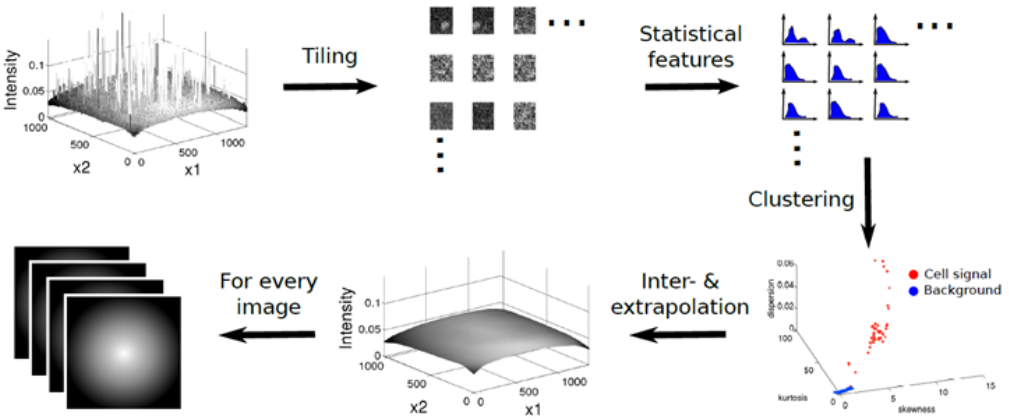

Figure 11 The procedure for the estimation of the time-dependent illuminated background $\boldsymbol{B(x;t)}$. The illustrated steps are applied to each image separately. First the images are split into overlapping sub-images. Then the moments of distribution of intensities are calculated for all tiles. Next a density-based clustering groups the tiles into a cluster with background tiles and a cluster with tiles containing cellular signal. A first background grid is constructed using the median intensity values of all background tiles. The final background $\boldsymbol{B(x;t)}$ is estimated by applying a two dimensional inter- and extrapolation to the initial grid. This process is applied to every image of the time-lapse experiment (Schwarzfischer et al., 2011) (Figure taken from (Schwarzfischer et al., 2011)).

2.3.3 Normalization of fluorescence microscopy images

According to different applications and image properties, QTFy provides various image normalization approaches. For simplicity, we keep the overall background level *b(t)* in our simulated images constant over time, leading to $B(x) \approx g(x)$. By dividing the image by the background all pixels will be distributed around 1.

Cellular signal will be normalized after a subtraction of 1 (Schwarzfischer et al., 2011)

$$s(x,t) = \frac{I(x,t)}{B(x,t)} - 1 \tag{12}$$

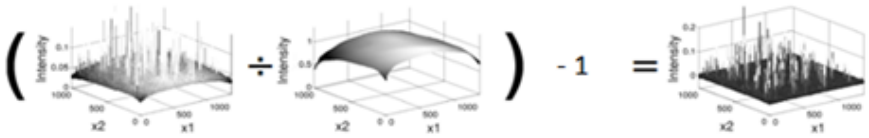

Figure 12 Image normalization for images with uneven illumination and a constant autofluorescence (Figure adapted from (Schwarzfischer, 2013))

2.4 Segmentation and quantification

After the simulated images are normalized, the cells are segmented and quantified by QTFy. In this section the two segmentation approaches, which are used by QTFy, are introduced briefly (Schwarzfischer, 2013) For details see (Otsu, 1979; Petrou & Petrou, 2010).

The first is a thresholding based method and is used to find the correct cell outline. In this process, a intensity threshold is allocated, which divides the image into pixels of foreground and pixels of background. Pixels above the allocated threshold are assigned to the foreground pixels, all other pixels are assigned to the background pixels. In gray level images, this can simply be done with Otsu's thresholding method, which detects the optimal threshold (Otsu, 1979).

The second segmentation approach, is the watershed method and is used to distinguish clumped objects, which cannot be achieved by thresholding. This method is performed by finding a seed pixel for each object and assigning neighboring pixels to the object, until all pixels of the image are assigned (Petrou & Petrou, 2010).

After the cell segmentation is done, the single cell quantification can be performed by summing up the intensities of all pixels within the segmented cell boundaries.

2.5 Statistical measures

2.5.1 Root-mean-square deviation (RMSD)

The differences between predicted or reference values and the values, which are observed can be described by the root-mean-square deviation ($RMSD$) (Martens & Martens, 2001).

The $RMSD$ is defined as

$$RMSD = \sqrt{\frac{\sum_{c=1}^{n_c}(i_{\mathrm{s},c} - i_{\mathrm{m},c})^2}{n_c}} \tag{13}$$

where n_c is the number of cells, $i_{s,c}$ is the simulated fluorescence intensity of cell c and $i_{m,c}$ is the measured fluorescence intensity for cell c.

As this deviation is scale-dependent and more informative with respect to the total values, the $RMSD$ is normalized to the mean of the simulated fluorescence intensities.

This normalized $RMSD$ (rn) can be calculated as (14)

$$rn = \frac{RMSD}{\frac{\sum_{c=1}^{n_c}(i_{\mathrm{s},c})}{n_c}} \tag{14}$$

2.5.2 Peak-signal to noise ratio (PSNR)

To assess the similarity of two images the peak-signal to noise ratio (PSNR) is a common measure (Bovik, 2002; Wang, Bovik, Sheikh, & Simoncelli, 2004). The PSNR is based on the mean square error, which is calculated for each pixel of the original image $I(x1, x2)$ and the corresponding pixel of the comparative image $K(x1, x2)$

$$MSE = \frac{\sum_{x1=0}^{m-1}\sum_{x2=0}^{n-1}[I(x1,x2) - K(x1,x2)]^2}{m \cdot n} \tag{15}$$

On this basis the PSNR is defined as

$$PSNR = 10 \cdot log_{10}\left(\frac{MAX_I^2}{MSE}\right)$$

$$= 20 \cdot log_{10}\left(\frac{MAX_I}{\sqrt{MSE}}\right) \tag{16}$$

where MAX_I is the maximum possible intensity of a signal in the image I (Bovik, 2002). The resulting value is of range $[0, \infty[$ (Kang et al., 2011). The better the similarity between two pictures, the higher is the $PSNR$. For identical pictures the $PSNR$ is not defined, as the MSE is zero in this case. (Burosch, 2014). Figure 13 illustrates the image quality or similarity at a high and a middle high $PSNR$.

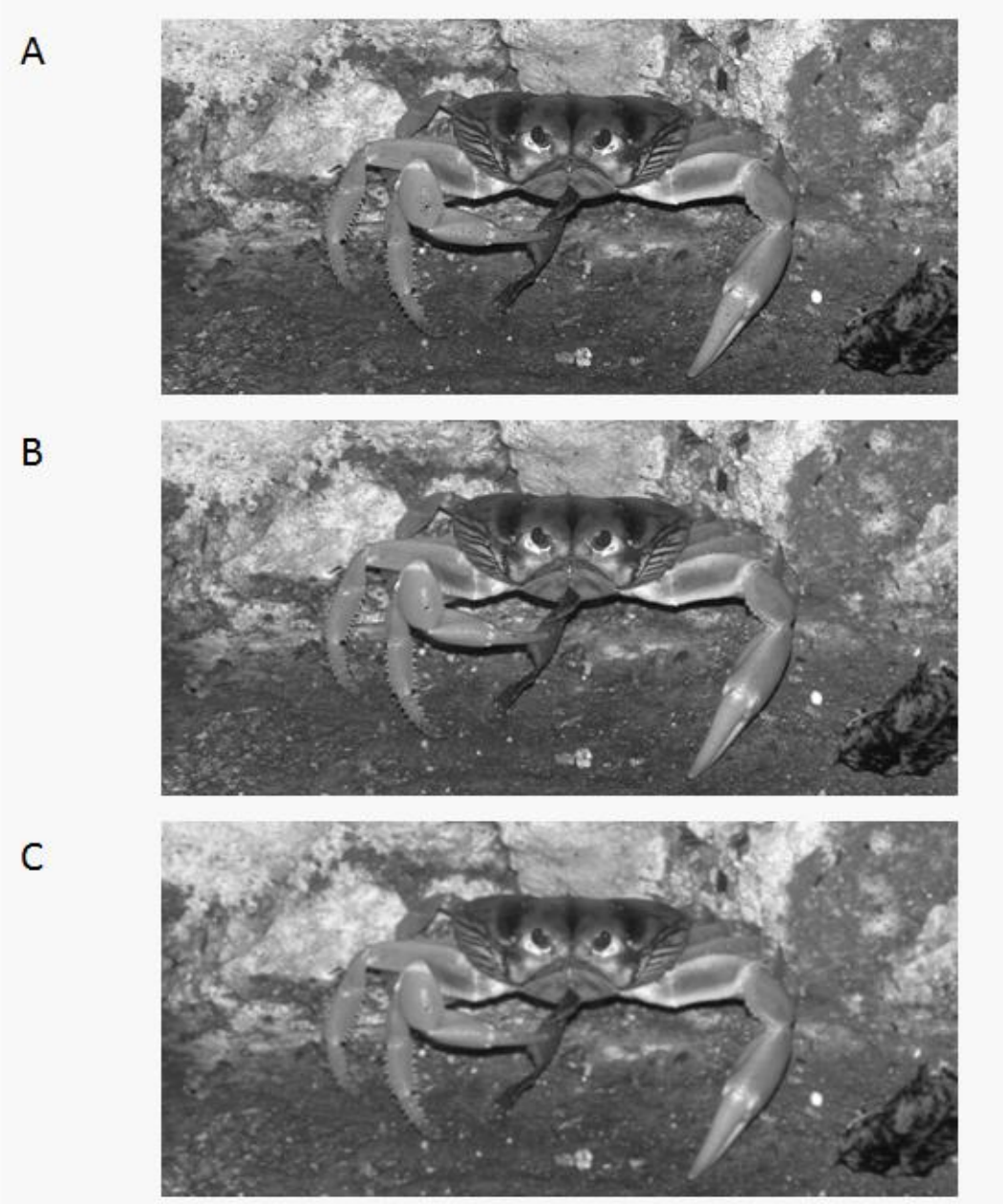

Figure 13 PSNR can be used to give a qualitative measure for image comparison. (A) Original image. (B) This images shows degradation due to compression artifacts. Its PSNR of 30 is commonly regarded as a good value. (C) This images represent a compression of the original image with lower quality setting, which results in a lower PSNR of 17.

3 Pipeline for the evaluation of QTFy

3.1 Description

In this Chapter, the created pipeline for the evaluation of the single cell quantification tool QTFy is described. Figure 14 represents an overview of the main steps. Details of the applied methods are explained in Chapter 2.

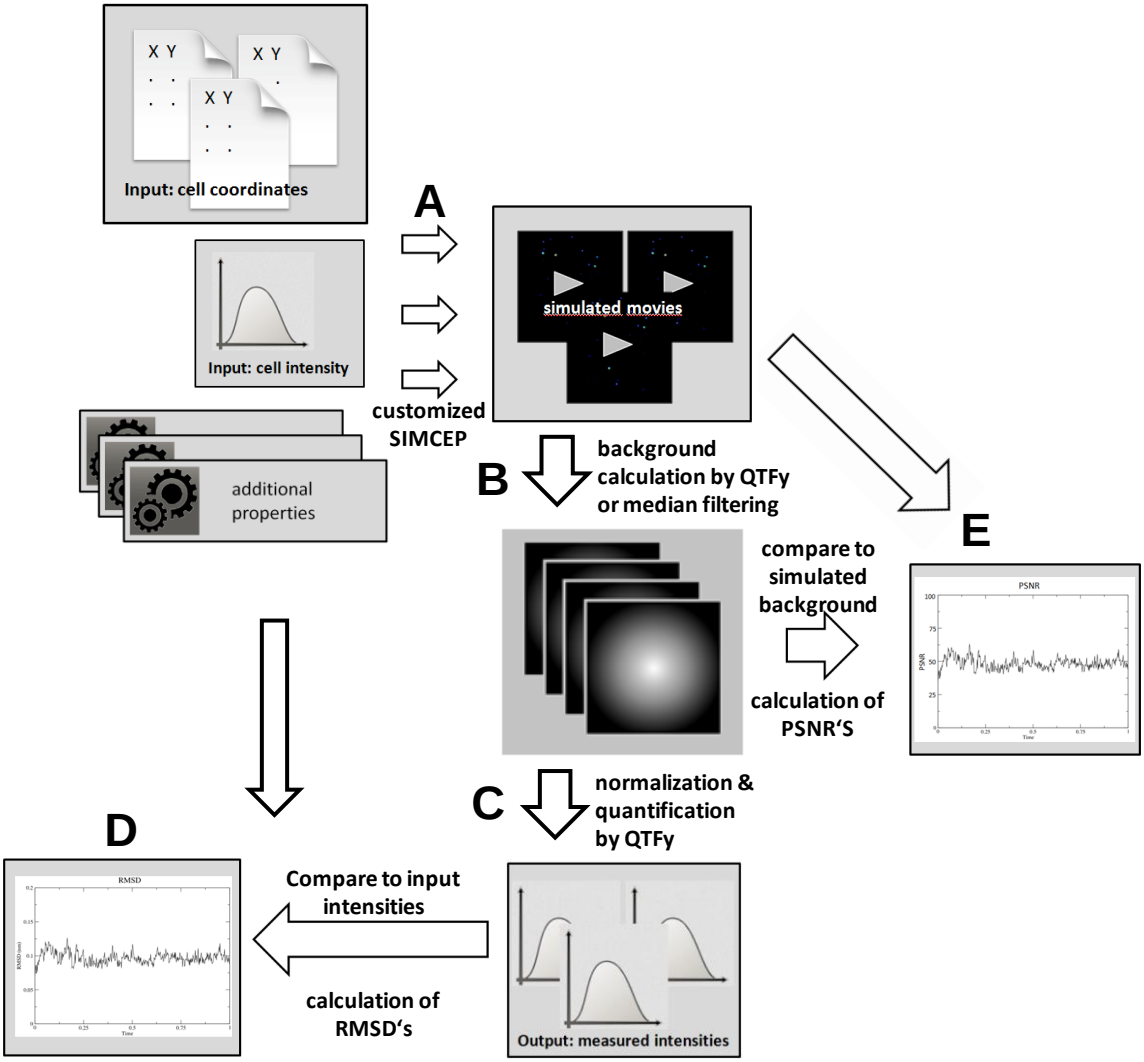

Figure 14 Workflow of the evaluation pipeline. (A) A customized version of SIMCEP is used to generate simulated fluorescence microscopy movies with known cell intensities (see Chapter 3.1.1). (B) Either a median filtering method (2.3.1) or the background estimation method by QTFy (see Chapter 2.3.2) is used to estimate the background of the simulated images (see Chapter 3.1.2). (C) QTFy normalizes the images and quantifies the cells. (D) The measured cell intensities are compared to the input intensities by calculating the RMSD (see Chapter 2.5.1 and 3.1.3). (E) The estimated backgrounds are compared to the simulated backgrounds by calculating the PSNR (see Chapter 2.5.2)

3.1.1 Simulation of fluorescence time-lapse microscopy movies

The first step is the simulation of the fluorescence time-lapse microscopy images. As mentioned above we adapted the image simulation tool SIMCEP (described in Chapter 1.3.2 and Chapter 2.2) to our purposes.

For the generation of single images, the following input parameters can be set by the user:

- Cell coordinates x
- Cell intensity i
- Cell radius r resulting in area A
- Number of cells c
- Illumination can be added with the taken illumination factor f
- Poisson noise can be added, its parameter is deduced from the illumination

To simulate synthetic microscopy movies instead of images, we extended the original version of SIMCEP to include the possibility to input cellular coordinates. For the generation of a movie, the user has to provide a character separated values (CSV) file, in which each line contains the semi-colon separated $x1$- and $x2$-coordinate. These coordinates represent the movement of the cell to be quantified, as for each entry an image is generated. We set the size of the image to 1000x1000 pixel.

Since the cell's position is already known for each time point, they do not have to be tracked anymore.

The intensity can be entered by the user as an arbitrary function, which is evaluated at every time point t. The maximum intensity is scaled to one by dividing every intensity by the highest calculated value.

The cell texture depends on the fluorescent light emitting characteristics of a cell. To simplify the evaluation of the quantification procedure, we adjusted the calculation of the texture provided by SIMCEP. We exclude any random parameters from the texture calculations and synthesize cells with a consistently bright texture. This means, that all pixels of one cell have the same grayscale-value based on the intensity function. Originally the nuclei are illustrated in blue in SIMCEP. We restricted the simulated images to gray scale values in 16-bit unsigned integer format. The brightness of each pixel is between zero which is black and 1, which is white. Multiplied by the number of pixel of the cell, we obtain the exact overall cell intensity, which can later be compared to the analysis results of QTFy.

To evaluate the performance of QTFy it is sufficient to focus on the cell's nucleus. Therefore our images do not include cytoplasm or other cell compartments but the nucleus. In the following the word cell is synonymously used for nucleus.

The size of the cell can be determined by specifying the cell radius. The default value of this parameter is five pixels, which is comparable to cells in real images. However, the same radius of cells does not lead to the exact same number of pixels within the cell, because the

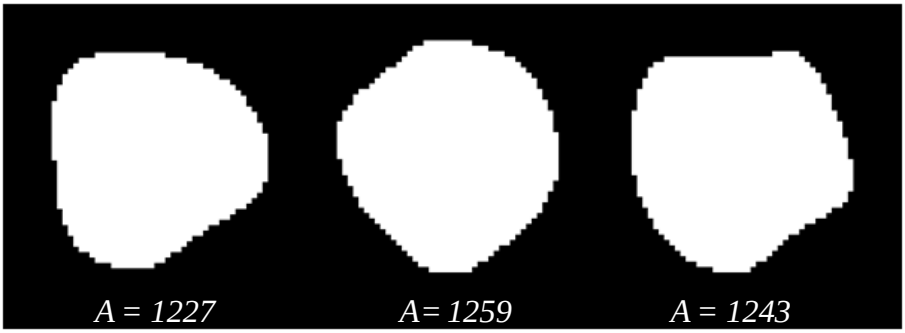

Figure 15 An initial fixed cell radius $\boldsymbol{r}$ does not lead to the same number of pixels $\boldsymbol{a}$ of a simulated cell and therefore not to the same total cell intensity. Here we see three different cells with $\boldsymbol{r = 20}$ pixels and slightly varying shapes and areas $\boldsymbol{A}$.

calculation of the shape is done separately for each cell and includes a random component (see Chapter 2.2.1). This also leads to differences in the overall intensities of cells with same radius and pixel intensity. In our pipeline we fix a and b of Equation ((6) and (7)) to 0.1, which results in faintly irregular shapes (see Figure 15).

To simulate a cell population, the user can set the number of cells to be displayed in the images. When no number of cells is entered only the cell with the specified coordinates is simulated. Additional cells will be randomly distributed over the image and will not be quantified by QTFy within this pipeline. For background simulation, the user can activate the simulation of an illumination function and specify the illumination factor f, which is used to scale the illumination matrix multiplicatively (see Chapter 2.2.2). The larger f is, the brighter the images will be. The illuminated background is adapted to the cell image as described in Chapter 2.2.2 and represented in Equation (8). Moreover, the user can include Poisson distributed noise in the simulation (see Chapter 2.2.3). Figure 16 exemplary shows an illuminated background and the corresponding background with Poisson noise.

With these parameters the synthetic fluorescence microscopy images can be generated (compare Figure 14 step A). Additionally, the pure background images, without cells and noise are stored and serve as ground truth for the later evaluation. Moreover a CSV file containing the coordinates of the cell to be examined, which correspond to the input coordinates, and the corresponding overall cell intensity, which is the input intensity multiplied by the number of pixels of the cell is generated.

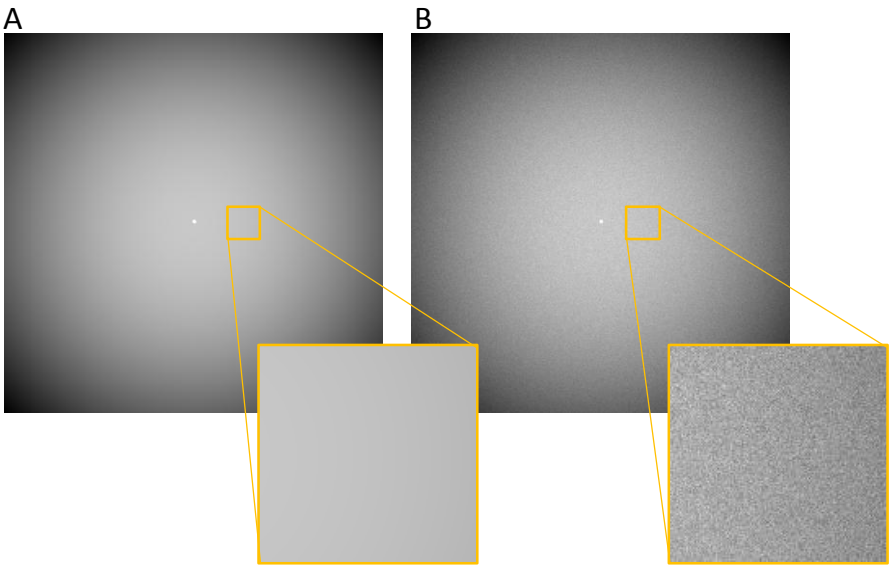

Figure 16 Simulated images with uneven illumination. (A) The image is simulated with an illumination factor of 10. (B) This image has the same illumination as (A) and Poisson noise is added.

3.1.2 Background calculation and cell quantification

The next step (compare Figure 14 step B) in the evaluation pipeline is the calculation of the background for every image, if required (images without illumination and noise do not need any background correction).The user can choose between the background estimation of QTFy (see Chapter 2.3.2) and the median filtering method provided by MATLAB (Mathworks, 2014c) (see Chapter 2.3.1). Both can be used to estimate the illuminated background of a fluorescent microscopy image and will be compared later (see Chapter 4.1.6).

For the median filtering a window size of 21 x 21 pixel is used for calculating the background image, as we need to remove not only the Poisson noise but also the cells of the image. This works well for images containing no bigger cells than the window size. The output of this step is a folder containing one background image for each image of the simulated time-lapse movie.

If the backgrounds were calculated in the previous step, either by QTFy itself or by median filtering, QTFy normalizes the images using Equation (12) (see Chapter 2.3.3). Afterwards QTFy performs the cell segmentation and quantification (see Chapter 2.4) (see Figure 14 step C). Finally, QTFy generates a file containing, among other parameters, the measured cell intensity for each time point (see Figure 14 step C).

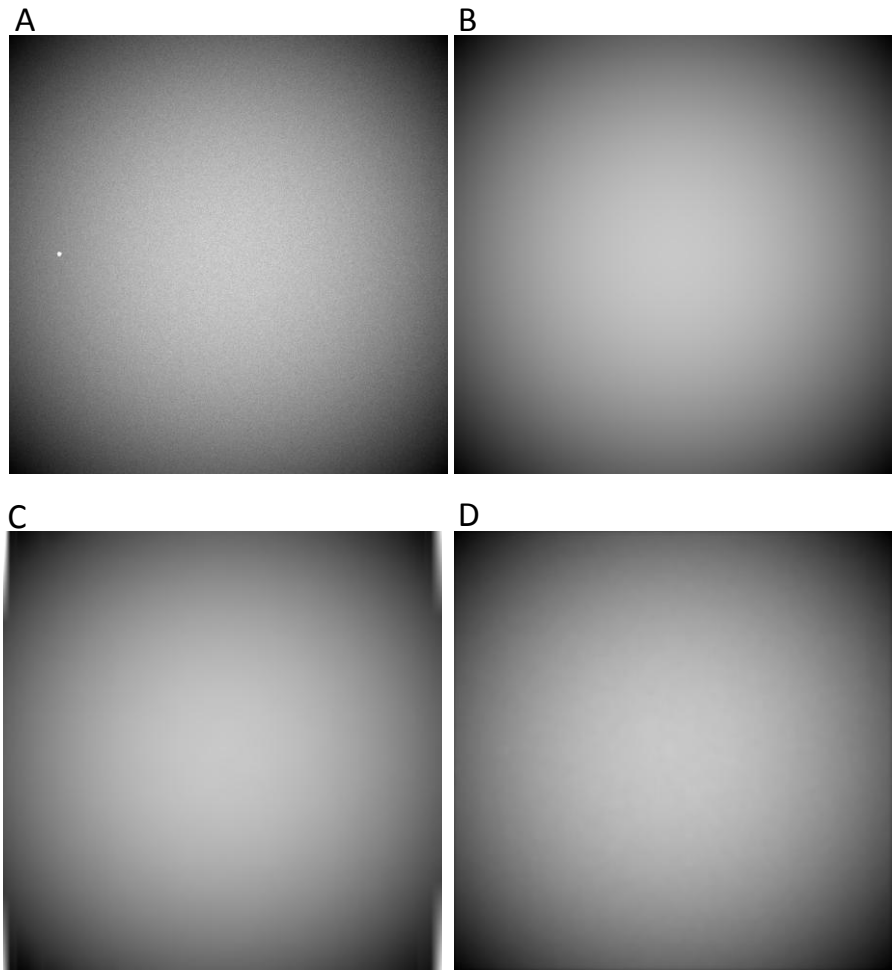

Figure 17 Exemplary representation of the different Background estimation method. (A) Original simulated microscopy image with one cell, uneven illumination and noise. (B) Original Background before the cell and noise were added. (C) Background estimated by QTFy from image (A) with a PSNR of 27 (see Chapter 2.3.2). (D) Background estimated by median filtering from image (A) with a PSNR of 40 (see Chapter 2.3.1).

3.1.3 Output

For each experiment the user can determine not only one input file with cell coordinates, but a set of CSV-files. So we have for each experiment a set of different cell movements, which all have a common intensity function. The previous described steps will be executed for each inputted file (see Figure 14 steps A to C). This results in a set of intensities for every time point measured by QTFy. The next step is to compare the measured intensities to the simulated intensities. This is done by calculating the RMSD (see Chapter 2.5.1) for each time point. Therefore Equation (13) is used, here n_c is the number of generated time-lapse movies and with Equation (14) the value is normalized by the average cell intensity for all time points (compare Figure 14 D).

If background images were estimated, an additional calculation is made within the pipeline (see Figure 14 E). To assess the quality of the calculated background images the PSNR (see Chapter 2.5.2) is calculated for each estimated background and the corresponding simulated background. For this, we used the method 'psnr' provided by MATLAB (Mathworks, 2014e). The output of the statistics part is a file, which contains for each time point the calculated RMSD score and the average of the PSNR scores of this time point. Moreover these scores are automatically plotted using the plot function of MATLAB (Mathworks, 2014d) and stored as MATLAB figure and as PNG image.

3.2 Generation of test data

For this thesis we analyzed the impact of the following parameters on the quantification performance of QTFy:

- Cell intensity
- Illumination
- Noise
- Cell size
- Cell density
- Signal to background ratio

For each experiment, we started the pipeline with a set of six different cell movements:

- Movement A and B: The cells move linearly through the image
- Movement C and D: The cells are randomly distributed in each frame.
- Movement E and F: The cells movement is based on the random walk processes, beginning in the center of the image

Each generated movie consists of 99 images.

3.2.1 Simulated movements

For the generation of movement A we set the starting coordinates to $x1$ = 10 and $x2$ = 500, what is at the middle of the left border, as our image is of size 1000 x 1000 pixels. In each step we applied a random walk with a drift to the right (see Chapter 2.2.4), which sets the

coordinates of the next time point randomly in a radius of 5 pixels after 10 pixels were added to $x1$.

For the generation of movement B we used the same procedure, but started at the middle of the upper border and added 10 pixels to $x2$ instead of the $x1$ before the random walk (see Chapter 2.2.4) was applied.

For the simulation of movements E and F we set the starting coordinates to $x1 = 500$ and $x2 = 500$, which is the middle of the image and applied the random walk as described in Chapter 2.2.4 with independent random variables in a range of $-10\ to\ 10$ pixels for the $x1$- and $x2$ -coordinates.

3.2.2 Movie properties

To evaluate the quantification performance of QTFy we simulated fluorescence time-lapse microscopy movies with the properties listed in Table 1. Furthermore we generated movies with constant background intensity and noise, to investigate the influence of the signal to background ratio on the quantification performance of QTFy (see Table 2). The radius of our simulated cells is 5 pixels. This results in cells with an area of about 65 to 85 pixels, which corresponds to cells in real movie.

For the evaluation of the background estimation of QTFy, we calculated for the experiments 2 to 6 all backgrounds also with the median filtering method (see Chapter 2.3.1) and repeated the statistics (see Chapter 4.1.6 for results).

Experiment number	Number of generated movies	Cell intensity	Illumination factor	Poisson Noise	Cell radius in pixel	Number of cells	GB of generated data
1	10 x 6	0.1 - 1.0	-	-	5	1	0.053
2	1 x 6	t	-	-	5	1	0.005
3	14 x 6	0.5	1-50	-	5	1	9.15
4	14 x 6	0.8	1-50	-	5	1	9.15
5	14 x 6	0.5	1-50	✓	5	1	15.9
6	14 x 6	0.9	1-50	✓	5	1	15.9

Table 1 Overview over the simulated test cases. The experiments 1 to 7 were performed with the six different movements (described in 3.2.1). The movies for experiments 1 and 2 were simulated without any background fluorescence, illumination or noise. The cell intensity increases in experiment 1 for each set of movies (six movies with different movements) by 0.1. In experiment 2 the cell intensity increases linearly according to time point $\boldsymbol{t}$. (see Chapter 3.1.1 for details, for results see Chapter 4.1.2). For the experiments 3 to 6 the illumination factor increases for each set of movies. (from 1 to 10 in steps of one, from 10 to 50 in steps of 10) (For results see Chapter 4.1.3 and 4.1.4).

Experiment number		Number of generated movies	Cell intensity	Background intensity	Noise	Cell radius	Number of cells
9		20	0.03 - 0.6	0.3	✓	5	1
10		20	0.04 - 0.8	0.4	✓	5	1
11		20	0.05 - 1.0	0.5	✓	5	1

Table 2 Overview over the test movies for the evaluation of the influence of the signal to background ratio. The background intensity for each set of movies is constant. The cell intensity increases during each set of movies, so that the signal to background ratio is comparative between the three sets of movies (see Chapter 4.1.5 for results).

4 Evaluation

In this Chapter, we present and discuss the results of our investigations of the test cases described in Chapter 3.2 and two movie from two real data sets.

4.1 Simulated data

4.1.1 Application of the pipeline

In general our experiments consist of several sets of simulated movies, where one set includes six movies with similar cell intensities but different movements (see Chapter 3.2). For each of these simulated movies QTFy performs its single cell quantification (see Chapter 1.3.1 and 3.1). Figure 18 A exemplarily illustrates the quantification results of one set of movies from Experiment 1 (see Table 1), where the intensity of each cell pixel is 0.5. Within our pipeline, the percentage rn score for each time point is calculated for the six quantification results to the real cell intensity (see Chapter 3.1.3 and Figure 14 D). Figure 18 B shows the percentage rn plot, which was automatically generated and stored within the pipeline (see Chapter 3.1.3). The analyses in the following subchapters are based on these normalized RMSD scores (see Equation (13) and (14)), generated within the pipeline.

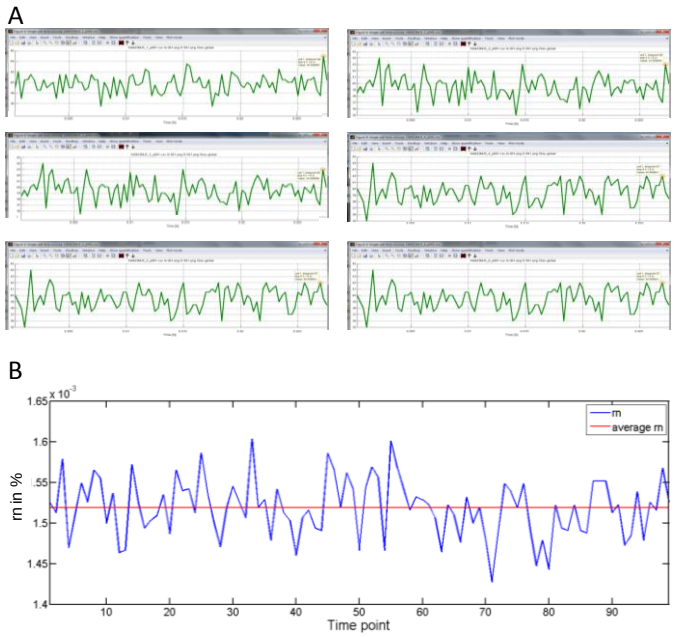

Figure 18 (A) Quantification results (intensity vs. time) of QTFy for one experiment. Most of our experiments (see Table 1) consist of several sets of simulated movies, where each set includes six movies with similar cell intensities and varying movements. These are the results of one set of movies from Experiment 1, with different movements and a constant cell intensity of 0.5. (B) Output of our pipeline and foundation of the following analyzes. Based on the quantification results of (A), these percentage $\boldsymbol{rn}$ scores were calculated (see Equation (13) and (14)) and plotted within the pipeline (see

Chapter 3.1.3). The cell intensity per pixel is 0.5 and the movies do not include simulated illumination or noise. The small deviations of about 0.00152 % in average are probably caused by rounding errors during the quantification process.

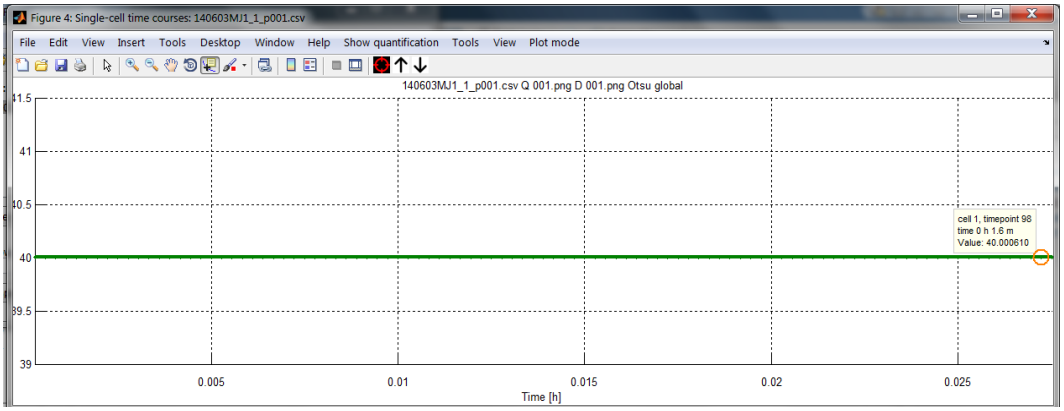

Figure 19 Quantification result of a movie with a constant cell intensity of 0.5 and a constant cell size of 80 pixels. This plot shows that the unsteady quantification results of other time courses, shown in this section (see Figure 18 for example) are not caused by quantification errors, but by the varying cell size of our simulated movies (see Chapter 3.1.1 Figure 15).

Figure 19 illustrates the quantification result of a movie, for that simulation, the random portion in the shape calculation was excluded. The cell has a constant intensity of 0.5 and a constant size of 80 pixels. As the movie was simulated without any background signal or noise, QTFy measured a constant and error free cell intensity of 40. Thus unsteady quantification results in Figure 18 and other, in this section presented, quantification results do not arise from quantification errors. They are caused by the varying cell size in our movies, due to a random portion in the calculation of the cell's shape (see Chapter 2.2.1 and Chapter 3.1.1, Figure 15).

4.1.2 Cellular intensity

To evaluate the quantification performance of QTFy with respect to cellular intensity we applied the tool to simulated movies without any background fluorescence, illumination or noise (see table 1 Experiment number 1), but varying cell intensities. The results are presented in Figure 20 and Figure 21 and were calculated as described in Chapter 3.1.3.

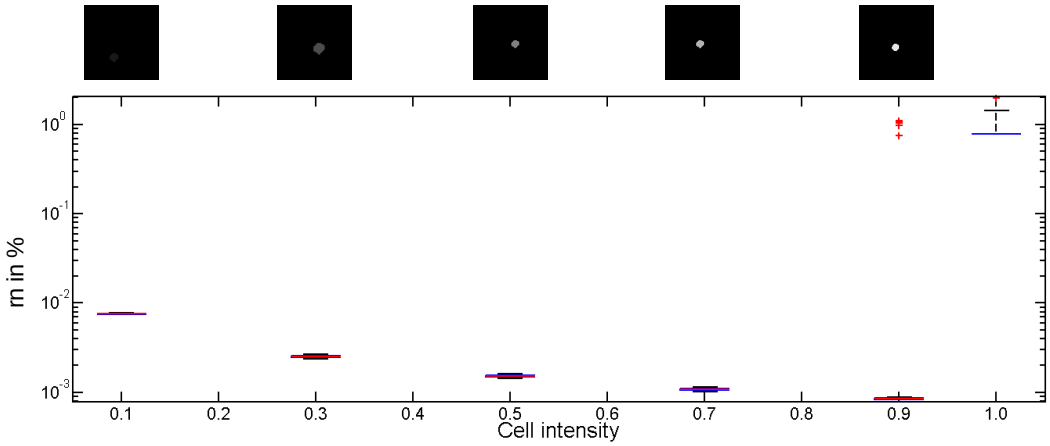

Figure 20 Influence of the cell intensity on the quantification performance of QTFy. Each box represents the percentage normalized RMSD scores $\boldsymbol{rn}$ for one set of movies with constant cell intensities and without background signal. For each time point, one $\boldsymbol{rn}$ score (see Equation (14)) was calculated (see Chapter 3.1.3 and Table 1). The red line on the boxes is the median and the lower and upper bound represent the 25 and the 75 percentiles, respectively. The vertical lines the box at cell intensity 1.0 extends to the most extreme data points. Additionally, outliers are marked by a red cross (Mathworks, 2014a). The images above the boxplot illustrate exemplarily the intensity of the cells in the corresponding movies.

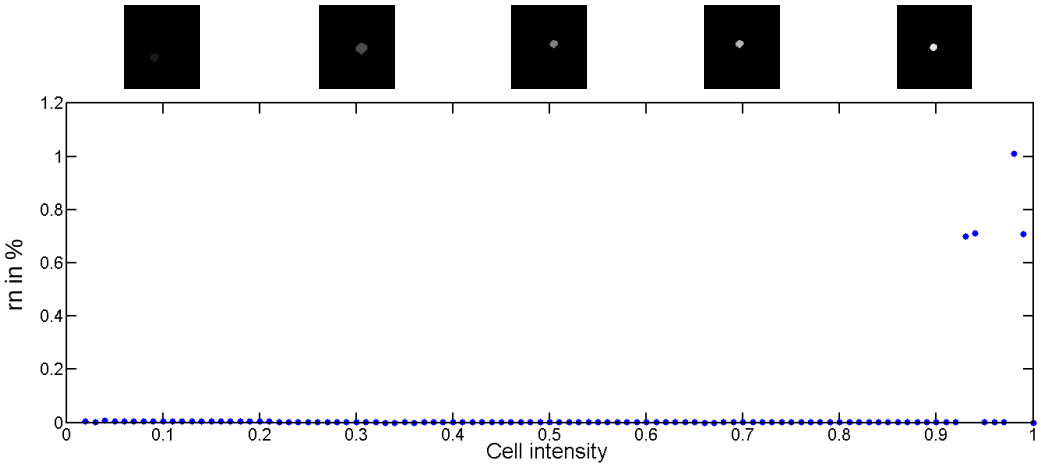

Figure 21 Influence of the cell intensity on the quantification performance of QTFy. This plot represents $\boldsymbol{rn}$ scores for each time point for a set of movies with increasing cell signal. The cell intensity increases linearly according to the time points.

In this simple scenario QTFy seems to make almost no errors ($RMSD \sim = 0$). For higher cell intensities (0.9 and 1.0) the RMSD scores increase, which implies that there are some measurement errors in the quantification. The results represented in Figure 21 confirm that the quantification works properly up to a value of 0.9.

The reason for the increasing RMSD scores for cells with intensities above 0.9 is that the cell segmentation misses single pixles that are located at the edge of the cell. Figure 22 shows the RMSD scores for the test movies with a cell intensity of 1. Three segmentation images from the cell inspector of QTFy are shown exemplary and demonstrate the reason for the deviations. This error stems from the internal segmentation process of QTFy. Images get blurred before thresholding by Otsu (see Chapter 2.4) resulting in less sharp edges of the cells. This blurring process normally helps to eliminate noise artifacts in real images but is counter productive in this artificial scenario.

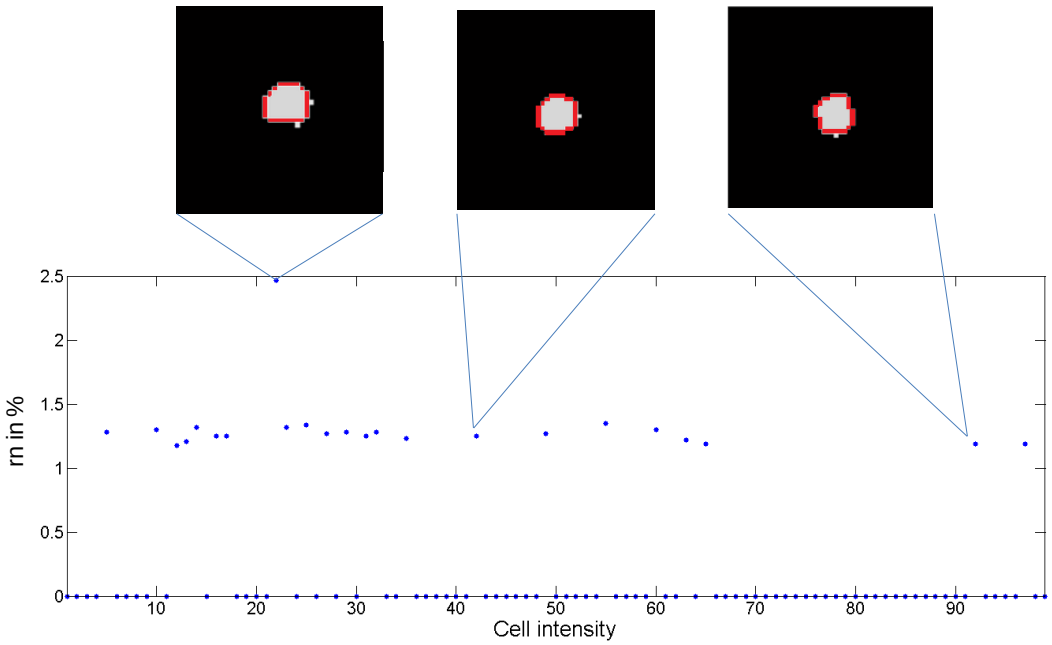

Figure 22 Quantification errors in movies with high cell signals are caused by a smoothing step in the segmentation process. The ***rn*** score for each time point of the test movies without background signal and with a cell intensity of 1.0 is plotted. For three time points the segmentation images illustrate the non-zero RMSD scores: Single pixels, that are located at the edge of the cell were missed in the segmentation.

4.1.3 Illumination brightness

For the evaluation of the influence of illumination brightness we simulated movies with varying brightness (see Table 1, Experiment number 3). These were generated with a constant cell signal of 0.5 and an increasing illumination factor and do not include noise. For the illumination factors 1 - 10 the movies are simulated with increments of one. For illumination factors 10 - 50, we simulated movies in steps of ten. Before the cells of these movies were quantified, the background of each image was estimated using the method of QTFy.

For movies including background signal the mean of the percentage rn is higher than for movies that do not include any background signal and image normalization (Figure 23). Except of a few outliers the RMSD scores are relatively constant up to a mean background signal of 0.42. For brighter illuminated images the RMSD score increase, meaning the quantification gets more inexact.

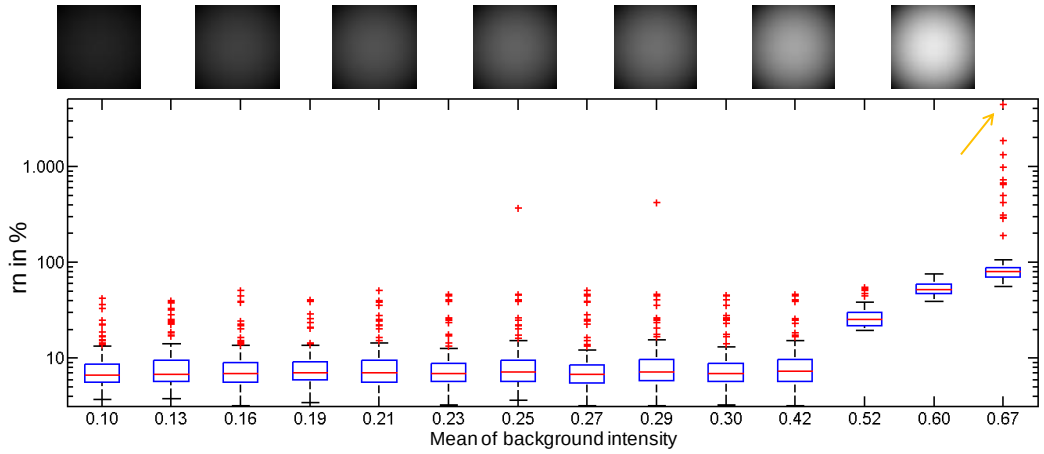

Figure 23 Influence of the illumination brightness on the quantification performance of QTFy. The boxes contain the ***rn*** scores calculated for each time point of the movies of experiment number 3 (see Table 1 and Chapter 3.1.3). Above the boxplot, representative images of the corresponding background images are shown. Figure 24 represents the corresponding cell to the outlier marked with the orange arrow.

We did this experiment once more with a different cell intensity (see Table 1, experiment number 4) and got similar results.

A big proportion of errors in the quantification of illuminated movies is caused by errors in the background estimation. Figure 24 shows a simulated image of one movie from Experiment 3, with a mean background signal of 0.67, and the corresponding background estimated by QTFy. These images are responsible for the largest outlier in the boxplot of Figure 23. At the edges of the background image considerable differences are visible due to the extrapolation step in the background estimation process (see Chapter 2.3.2). When a cell

is, like in this example, located in such an inaccurate area the normalization fails and wrong cell intensities are measured.

Figure 25 presents that for this example a very high quantification value is calculated. Due to the much too low estimated background in the area where the cell is located, the cell signal was divided by a very small value during the normalization (see Chapter 2.3.3) resulting in this high measured cell intensity.

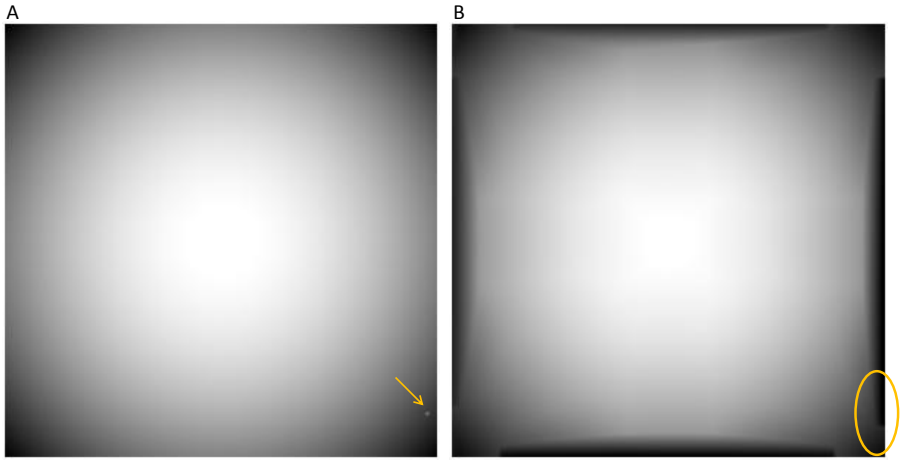

Figure 24 Exemplary illustration of the source of quantification errors in movies with illumination. (A) Simulated image with a cell intensity of 0.5 and a mean background intensity of 0.67 (illumination factor = 20). (B) Estimated background of the QTFy procedure based on (A). The cell of image (A) is located in a corner area (marked by the orange oval) of the background where estimation is false due to the extrapolation step. The resulting wrong image normalization leads to false fluorescence quantifications.

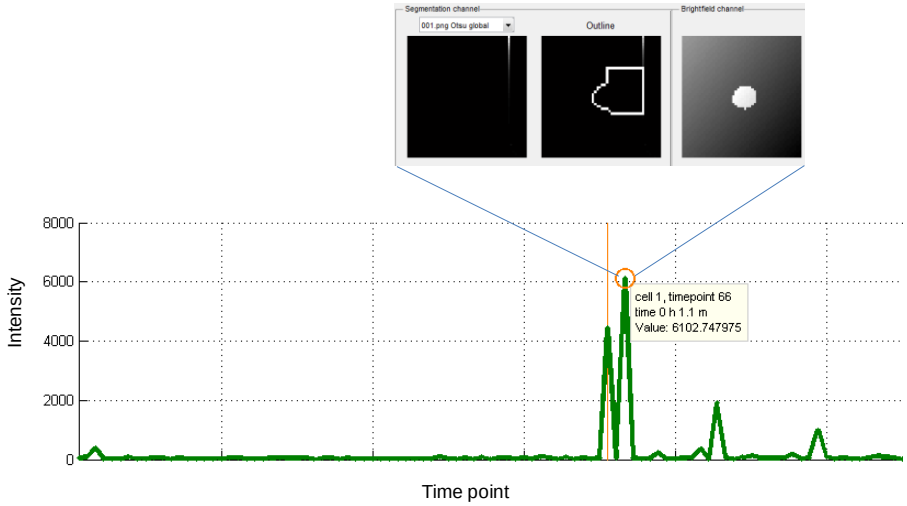

Figure 25 Quantification result for one movie of experiment 3 (see Table 1) with a mean background signal of 0.67 (see Figure 23). The marked peak is the value for the cell shown in Figure 24 (A). Above the peak, the corresponding segmentation image is shown. Errors in the background estimation (see Figure 24 (B)), result in a much too high quantification result with a percentage $\boldsymbol{rn}$ score of over 3000 % of the real cell intensity for this time point (see Figure 23).

4.1.4 Poisson Noise

In Experiment 5 we left all parameters of the simulated movies unchanged, compared to the movies in Experiment 3, but we added Poisson noise to the images (see Table 1 and Chapter 3.2 for details). In this way we want to assess the impact of noise on the quantification performance of QTFy.

Figure 26 illustrates the resulting percentage rn scores. In comparison to the results for illuminated movies without noise (see Figure 23), it can be seen that the mean values for all presented mean background intensities are at similar levels. But up to a mean background intensities of 0.42, there are in average one to two outliers with high percentage rn scores more than in the results for the illuminated movies without noise. However, the extreme high values for the brightest movies are absent.

Similar to the previous Section, we simulated this experiment for the impact of noise once again with a different cell intensity (see Table 1, Experiment number 6) and got similar results.

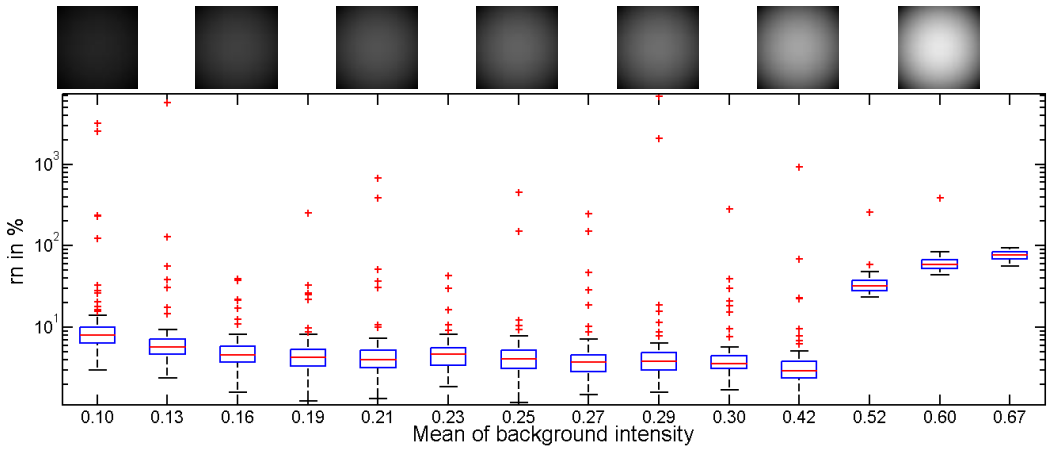

Figure 26 Influence of the noise on the quantification performance of QTFy. The boxes contain the percentage $\boldsymbol{rn}$ scores calculated for each time point of the movies of Experiment number 5 (see Table 1 and Chapter 3.1.3). Above the boxplot, miniature images of the correspond images, including the Poisson distributed noise are shown.

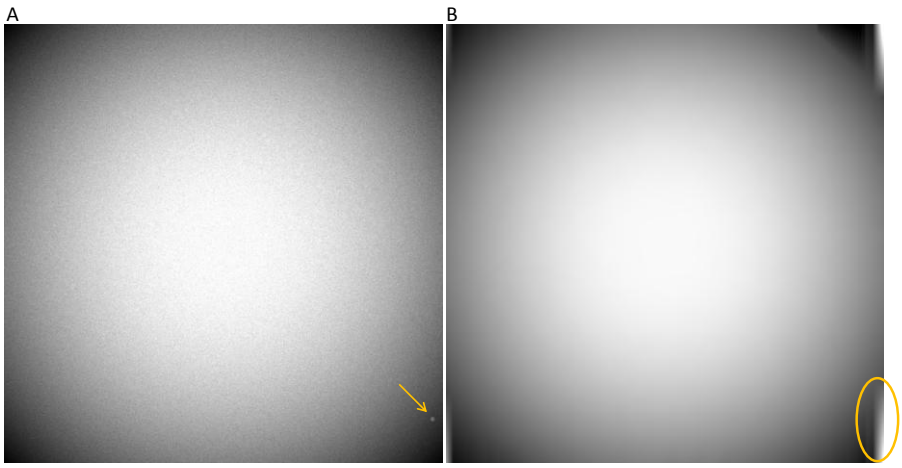

Figure 27 Illustration of the reason for the absence of the highest outlier of the boxplot in Figure 23. (A) Simulated image with a cell intensity of 0.5 and a mean background intensity of 0.67 (illumination factor = 20). These are the same parameter like in the image of Figure 24 (A), but this image contains noise. (B) Estimated background of the QTFy procedure based on (A). Compared to the cell in Figure 24, the cell of image (A) is here located in a area (marked by the orange oval) of the background where the estimation is less faulty.

4.1.5 Signal to background

To evaluate the influence of the cell signal to background ratio on the quantification performance, we created movies with different background intensities and increasing cell intensities adapted to the respective background signal. The background intensity is in each image constant and evenly distributed. Moreover all this movies contain simulated noise (see Table 2).

In general, Figure 28 shows that the RMSD decreases with higher signal to background ratios. Furthermore, the RMSD for each signal to background ratio decreases also with higher background intensities. The brighter a background signal is the lower is the corresponding RMSD compared to images with the same signal to background ratio. This can be explained by the fact that for higher background signal, the effect of the Poisson distributed noise is indeed rising, but the signal to noise ratio increases anyway (see Chapter 2.2.3 for details).

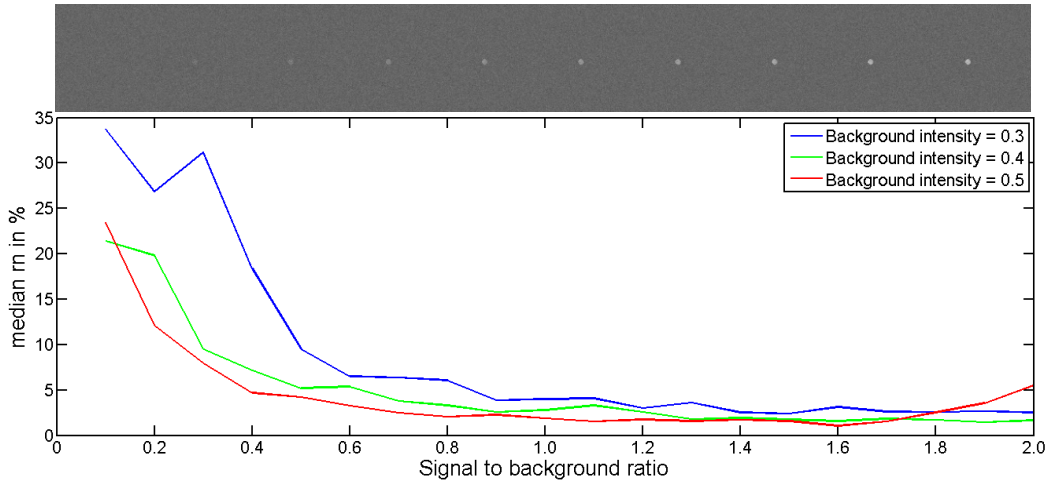

Figure 28 Influence of the signal to background ratio on the quantification performance of QTFy. Each line represent the median percentage $\boldsymbol{rn}$ scores for a movie with constant evenly distributed background signal, Poisson distributed noise and an increasing cell signal resulting in increasing signal to background ratios (see Table 2, Experiments 9 to 11). Above the plot exemplary images, with raising signal to background ratios, from Experiment 10 with a background intensity of 0.4 are shown.

4.1.6 Background estimation method

4.1.6.1 Comparison of quantification performance

To assess the performance of the background estimation of QTFy we repeated the Experiments 3 to 10 and replaced the calculation of the background from QTFy by the median filtering method from Matlab (see Chapter 2.3.1).

Figure 29 illustrates the resulting RMSD scores for the repeated Experiment 3 with the other background estimation method (compare to Chapter 4.1.3 and Figure 23). Compared to the results with the background estimation of QTFy, the mean RMSD is much lower for all mean background intensities and there are no extreme high outliers. From a mean background intensity of 0.42 the RMSD scores start to increase with the mean background intensity. For lower background intensities the scores are almost constant with one outlier per mean background intensity.

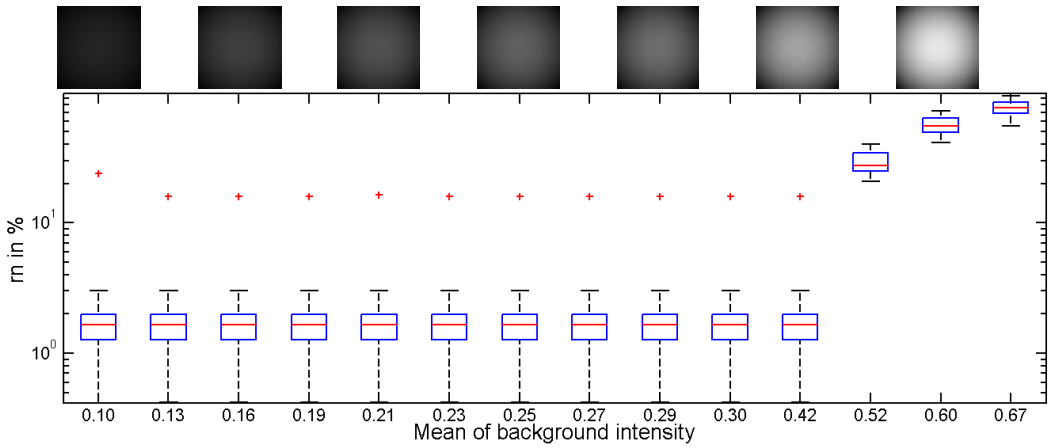

Figure 29 Influence of the illumination brightness on the quantification performance of QTFy. The parameters for the simulation of the movies are the same as described in Chapter 4.1.3. But for the normalization of the simulated images we used background images, which were calculated by the median filtering method (see Chapter 2.3.1) instead of the method of QTFy (see Chapter 2.3.2).

The outliers in Figure 29 are caused by a cell which is located in the left lower corner of the image, in one time point of one of the six movies. The corners of the images remain almost unaffected by illumination. Therefore the error is relatively constant over the different mean background intensities.

Figure 30 shows the segmentation image of this cell and is the reason for the quantification error. The cell was not correctly segmented which covered the cell only partly resulting in a very low quantification value (Figure 31). Figure 31 also illustrates that this is the only outlier of this simulated movie.

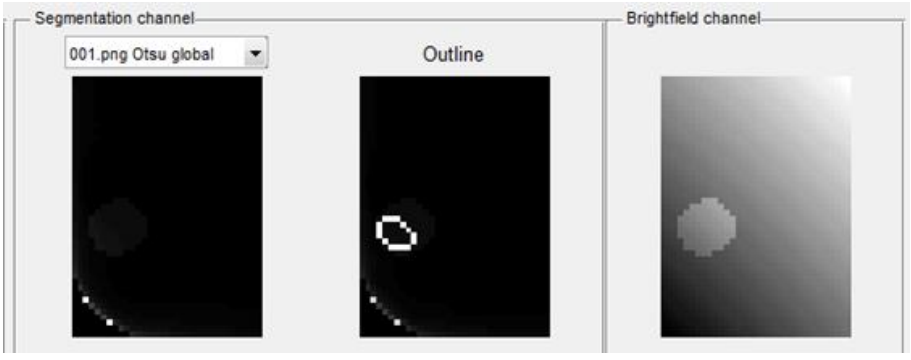

Figure 30 Illustration of the reason for the quantification errors in illuminated movies with background images calculated by median filtering. This cell is located in the left lower corner close to the edges of the image and was covered only partly during segmentation. This effect is probably also caused by an artifact of the normalization, due to estimation problems of median filtering in areas close to edges.

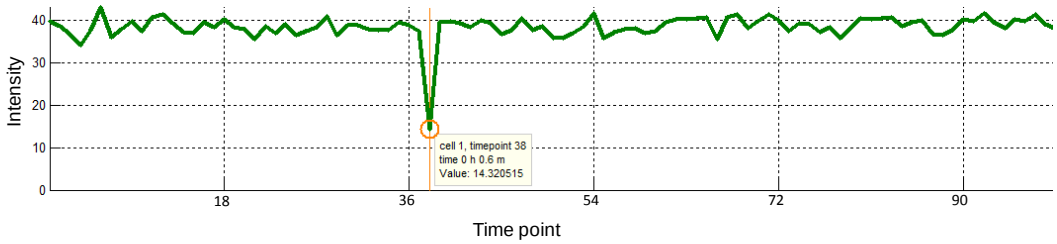

Figure 31 Quantification results for the movie containing the cell described in Figure 30. The result for the wrong segmented cell is marked by the orange circle and is the only outlier in the quantification results of this movie.

We did the same investigations with a cell signal of 0.8 instead of 0.5 and got similar results (data not shown).

For the same movies but with noise, we get a similar schema of RMSD scores as without noise. The scores starts to rise from a mean background intensity of 0.42.Images with lower mean intensity values have one outlier and rather homogeneously distributed RMSDs (see Figure 32). The Outliers are caused by the same images as the outliers in the Experiment without noise.

Compared to the corresponding results of the quantification based on backgrounds estimated with the QTFy method (see Chapter 4.1.4 and Figure 26) the mean RMSD scores of all mean background intensities are on a similar level, but the numerous outliers are absent, which indicates, that the median filtering is more steady in its performance for this simulated movie scenario.

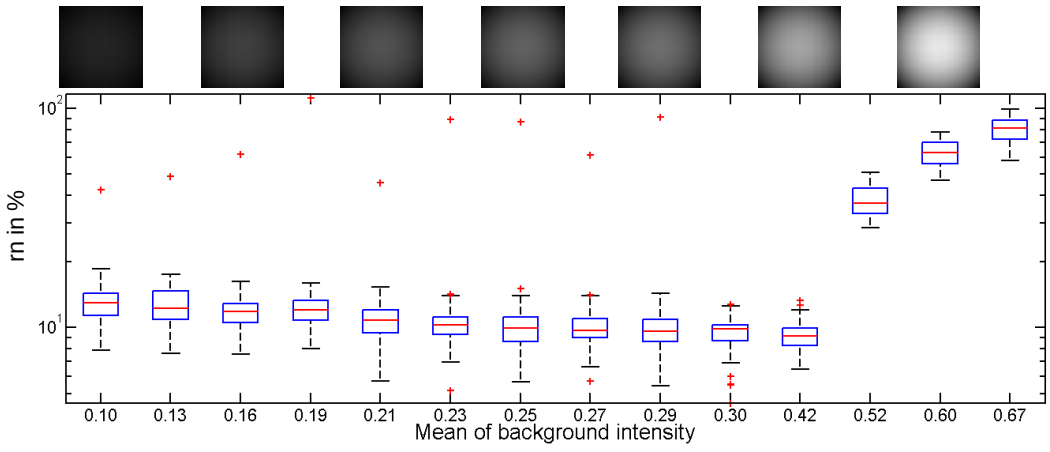

Figure 32 Influence of Poisson noise on the quantification performance of QTFy. The parameters for the simulation of the movies are the same as described in Chapter 4.1.4. But for the normalization of the simulated images we used background images, which were calculated by the median filtering method (see Chapter 2.3.1) instead of the method of QTFy (see Chapter 2.3.2).

As for the other experiments for the influence of noise or illumination we repeated this experiment with a cell intensity of 0.8 instead of 0.5 and got similar results.

Figure 33 summarizes the results of the influence of illumination and noise on the quantification performance of QTFy for both background estimation methods. Compared to the percentage rn scores for the backgrounds estimated by median filtering, the scores for the backgrounds of the QTFy method are higher. On average the quantification based on the backgrounds estimated by QTFy are lower for noisy movies, which can be explained by the fact that this background estimation works better for noisy images (see Chapter 4.1.6.2 and Figure 38).

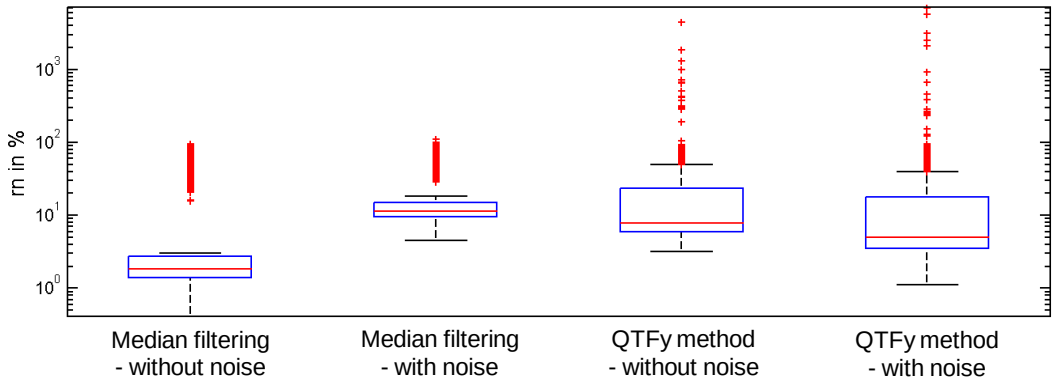

Figure 33 Overview over the influence of illumination and noise on quantification performance of QTFy. The boxes contain the percentage rn scores calculated for each time point of the movies of Experiments 3 and 5 (see Table 1). The backgrounds where once estimated by median filtering and once by QTFy. (see Figure 23, Figure 26, Figure 29 and Figure 32 for the detailed distributions of the scores of the Experiments).

4.1.6.2 Comparison of image similarity

We compared the estimated background images to the simulated background images by calculating the PSNR (see Chapter 2.5.2). We quantified the PSNR for both methods, the median filtering as well as the QTFy method for all Experiments to investigate the impact of illumination or noise.

Figure 34 and Figure 35 represent the results for the PSNR calculations for the Experiments with backgrounds estimated by the median filtering method. In both plots the PSNR scores decreases according to an increasing mean background intensity. The higher the PSNR score is the more similar are the compared images (for details see Chapter 2.5.2). That means the median filtering method performs better, the darker the image is. All boxes in the two plots are very narrow, which comes from the fact, that all estimated backgrounds of this method are very similar.

By comparing Figure 34 and Figure 35 it is obvious that the method works for noisy images worse than for less noisy images. For images without noise the method reaches scores between about 80 and 60. For noisy images however scores between about 52 and 42 (see also Figure 38), what still implies a high similarity of the compared images (see Figure 13).

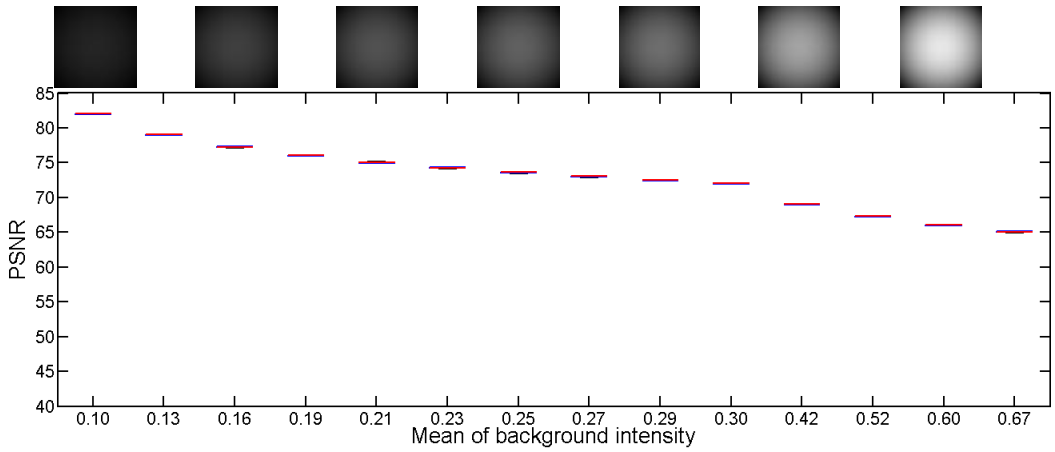

Figure 34 Similarity between simulated background images and by median filtering estimated backgrounds for movies with illumination and without noise. The PSNR scores were calculated (see Chapter 3.1.3) and plotted for each time point of the six movies in the Experiment 3 (see Table 1). The single boxes are very narrow, what shows, that the median filtering is very constant in its performance. The higher the mean background intensity is, the lower is the corresponding PSNR, what implicates that the method works more reliable for images with low mean background intensities.

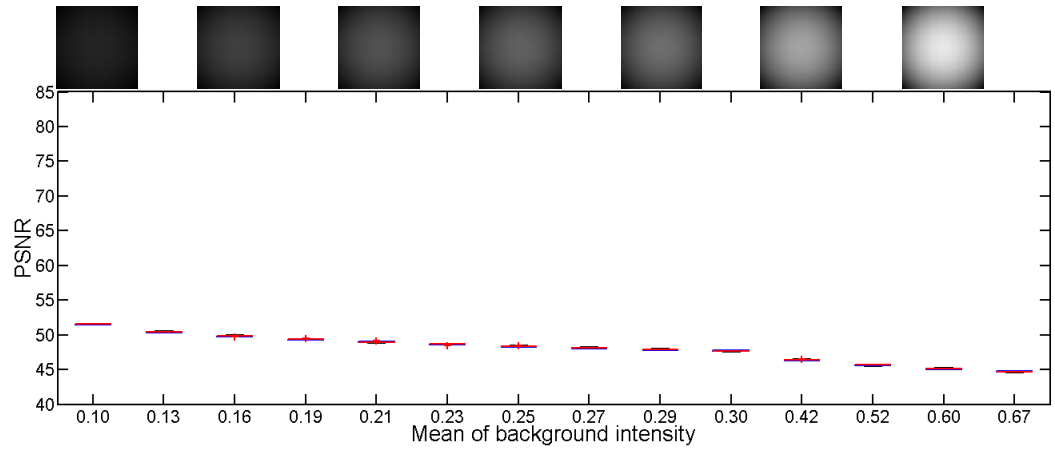

Figure 35 Similarity between simulated background images and by median filtering estimated backgrounds for movies with illumination and noise. The PSNR scores were calculated (see Chapter 3.1.3) and plotted for each time point of the six movies in the Experiment 5 (see Table 1).Compared to the scores of similar movies but without noise (Figure 34), the boxes are slightly less narrow and overall lower, what indicates, that the median filtering works less reliable for noisy images.

Figure 36 and Figure 37 show the PSNR scores for the Experiments that are based on the estimated backgrounds from QTFy.

For the movies with illumination but without noise (see Figure 36) the PSNR score also decreases according to an increasing mean background intensity. Just the score for the highest mean background intensity constitutes an exception. This PSNR score is higher than the scores for the previous three mean background intensities. Moreover, in contrast to the other results, the box for this intensity is much more compressed, which shows that the estimated backgrounds for these images are more similar to each other.

The PSNR score for the noisy movies (see Figure 37) are much more widespread for the single mean background intensities compared with the scores for illuminated images without noise (see Figure 38). But the mean is more constant and on average the scores are higher than the scores for the illuminated images without noise (see Figure 38). This means, that the background estimation method of QTFy works, in contrast to the median filtering, better for noisy images than for images without noise.

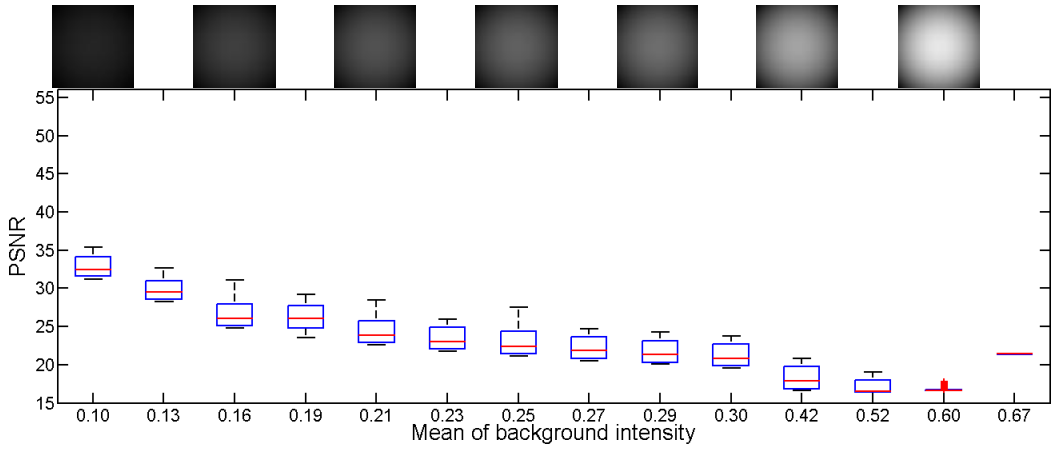

Figure 36 Similarity between simulated background images and estimated backgrounds by QTFy for movies with illumination and without noise. The PSNR scores were calculated (see Chapter 3.1.3) and plotted for each time point of the six movies in the Experiment 3 (see Table 1). The single boxes are not very narrow what shows that the background estimation is not constant in its performance. The higher the mean background intensity is, the lower is the corresponding PSNR, what implies that the method works more reliable for images with low mean background intensities. The box for the highest mean background intensity represent an exception. It is higher than the previous boxes and very narrow.

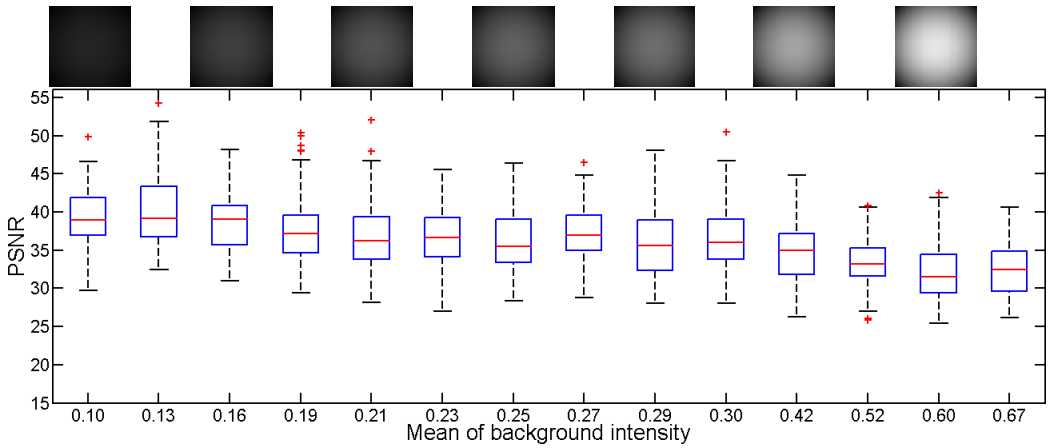

Figure 37 Similarity between simulated background images and by QTFy estimated backgrounds for movies with illumination and noise. The PSNR scores were calculated (see Chapter 3.1.3) and plotted for each time point of the six movies in the Experiment 5 (see Table 1). The PSNR scores for each mean background intensity in this experiment are very widespread, but in the mean they are constant over the different background intensities.

Figure 38 clearly shows that the median filtering method leads, in these test scenarios, to estimated backgrounds with higher similarity to the simulated images, than the used estimation method of QTFy. This effect can probably be explained by the falsely extrapolated regions of the estimated background images and could be reduced by optimizing the features and parameters used in the clustering algorithm (see Chapter 2.3.2)

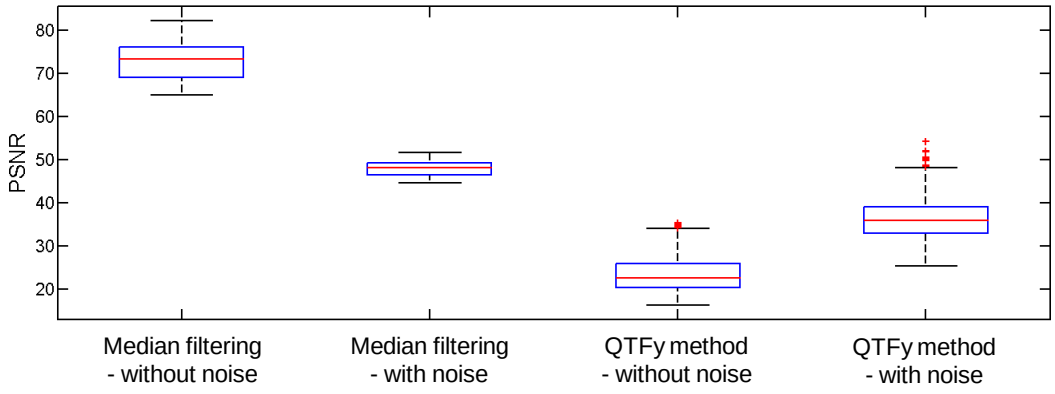

Figure 38 Overview over the similarity between simulated and estimated backgrounds. The boxes represent the calculated PSNR scores for illuminated movies with and without noise (see Table 1, Experiment 3 and 5). For each time point of these movies, the background was compared to the estimated background by median filtering and QTFy, respectively. For both test scenarios, the median filtering reveal higher image similarity than the method of QTFy. In contrast to the median filtering, the method of QTFy reveals higher PSNR scores for noisy images, than for illuminated images without noise.

4.2 Real data

With our analyses we have the possibility to assign a real fluorescence microscopy movie to a certain percentage $RMSD$, which is normalized to the simulated cell intensity (rn) (see Chapter 2.5.1, equation (13) and (14)) range, based on the respective movie properties. We exemplary quantified the intensities of cells in two different fluorescence microscopy movies. Both data sets have been generated in the lab of Timm Schroeder (BSSE Basel, ETH Zurich) and quantified at the ICB, Helmholtz Zentrum München.

4.2.1 Time-lapse microscopy movies of hematopoietic stem and progenitor cells

The first movie consist of ten frames of differentiating blood stem cells (Orkin & Zon, 2008) with a fluorescently tagged PU.1 transcription factor protein (Krumsiek, Marr, Schroeder, & Theis, 2011). The sizes of the cell in the different time points are comparable to the cell sizes in our previously described experiments (see Chapter 3.2.2). Furthermore the movie is unevenly illuminated and includes noise (see Figure 39 (A)). Using QTFy, we estimated the background for each of the ten frames and executed the fluorescence intensity quantification for one of the cells (see Figure 39 (B)). The estimated backgrounds have a mean pixel intensity of 0.39. Considering the analysis results of the influence of noise to the performance of QTFy (shown in Figure 26) and the present movie properties, we can assign an average rn of $4.23\% \pm 7.24\%$ to the quantification of nuclear fluorescent protein intensity (see Figure 39 (C)).

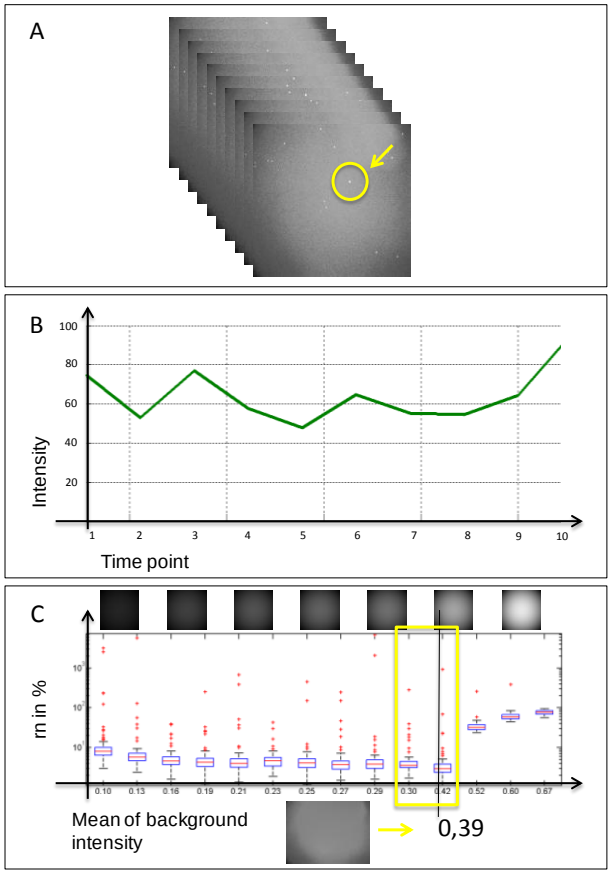

Figure 39 Exemplary fluorescence intensity quantification for a real movie with blood stem cells. (A) Fluorescence time-lapse microscopy movie with uneven illumination, noise and a mean background intensity of 0.39. (B) Corresponding quantification result of QTFy for the yellow marked cell in (A). (C) Analysis results for simulated movies with uneven illumination and Poisson noise (see Chapter 4.1.4). The yellow box marks the approximate position where the movie (A) can be assigned according to its characteristics.

4.2.2 Time-lapse microscopy movies of pluripotent embryonic stem cells

The second movie shows embryonic stem cells (Chambers et al., 2007) in LIF serum, where the pluripotency transcription factor Nanog has been tagged with yellow fluorescent protein (Filipczyk et al., 2013). The mean intensity of estimated backgrounds is 0.17. The other movie properties are similar to the first investigated movie and we can also assign an average rn of about $5.9\% \pm 5.6\%$. Figure 40 represent the second movies with its quantification results and the corresponding analysis results.

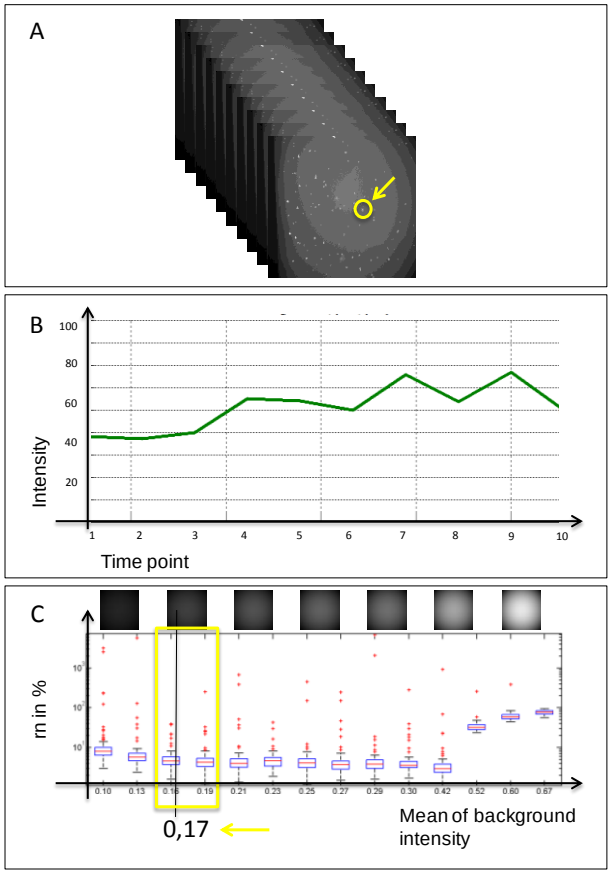

Figure 40 Exemplary fluorescence intensity quantification for a real movie with embryonic stem cells. (A) Fluorescence time-lapse microscopy movie with uneven illumination, noise and a mean background intensity of 0.17. (B) Corresponding quantification result of QTFy for the yellow marked cell in (A). (C) Analysis results for simulated movies with uneven illumination and Poisson noise. The yellow box marks the approximate position where the movie (A) can be assigned according to its characteristics.

5 Summary and outlook

Within this thesis we created a pipeline for the evaluation of the single cell quantification tool QTFy. In the pipeline fluorescence time-lapse microscopy movies are simulated, using a customized version of the simulation tool SIMCEP. Subsequently, QTFy performs its quantification and the results are compared to the simulated cell intensities. The results were assessed by calculating the percentage $RMSD$ normalized to the simulated cell intensity (rn).

We applied the pipeline to seven different test scenarios with a total number of 462 movies to investigate the influence of these seven factors on the quantification performance of QTFy. Moreover, we have examined the performance of QTFy's background estimation method, by calculating the background of 336 of the 462 simulated movies with the median filtering method. We used these estimated backgrounds to normalize the movie images, repeated the quantification and compared the results to the previously obtained ones. In addition we evaluated the similarity between the estimated background images of both methods and the simulated backgrounds and compared these results.

On movies without any background signal or noise QTFy performs very well. The only quantification errors have occurred for cells from a pixel intensity above 0.9, which have single pixels on the edge of the cell. These single pixels were missed during the segmentation step of QTFy, leading to small deviations in the quantification results. Typically noise occurs in real microscopy images. To eliminate the noise, the images get blurred during the segmentation, resulting in less sharp edges. In this artificial test scenario, this process leads to the mentioned segmentation errors.

For experiments based on backgrounds, estimated by QTFy, we found out that there are only small differences between the quantification performance for movies with illuminated background with and without noise. For noisy images the average rn is actually slightly lower, than for illuminated movies without simulated noise. Which means that the quantification performs slightly better for illuminated movies, which contain noise, than for illuminated movies without noise. In both scenarios (illuminated movies with and without noise), the brightness of illumination has only a negative effect on the quantification results from a mean background intensity above 0.42. The mean rn increases rapidly above this mean background intensity. The noisy movies have many outliers with high RMSD scores up to the limit value of 0.42, whereas the movies without noise have more of such outliers for the movies with high mean background intensity. In all tested scenarios, the high percentage rn outliers are caused by wrongly estimated background areas.

For the same two scenarios, we performed the quantification based on backgrounds estimated by a median filtering method. We got comparable results, but the high outliers of percentage rn scores were absent and in contrast to the results based on the backgrounds estimated by QTFy, the quantification performed better for the movies without noise.

We investigated also the influence of the signal to background ratio on the quantification performance and found out, that the higher the signal to background ratio, the better is the

quantification performance. Moreover, the brighter the background is, the better is the quantification performance compared to images with similar signal to background ratios. This effect is caused by the decreasing impact of the Poisson distributed noise for increasing background intensities.

All in all the median filtering method leaded to better quantification results, than the background estimation method of QTFy, which we confirmed by demonstrating, that the backgrounds estimated with this methods have a higher similarity to the simulated backgrounds than the estimated backgrounds of QTFy. This effect can probably be explained by the false extrapolated regions of the estimated background images and might be reduced by optimizing the features and parameters used in the clustering algorithm. However, the background estimation method by QTFy is developed for real microscopy movies, which normally contain a larger number of cell. We assume, that for such movies the median filtering do not perform adequate, and the QTFy method outperforms it.

To confirm this assumption, the pipeline can be used to generate movies with an increasing number of cells within the movies. The runtime for the generation of the movies scale up according to number of cells within the images. Therefore the distributed SIMCEP version is unsuitable for a large number of experiments or long movies with many cells. To investigate movies, that contain many cells, the runtime should be reduced, by implementing a more efficient solution.

Moreover the following modifications could be performed to simulate more realistic fluorescence time-lapse microscopy movies.

- simulate gene expression (in the cell intensity)
- adapt the texture to realistic cells (not every pixel has the same intensity)
- simulate more detailed cells (not only the nucleus but cytoplasm and sub cellular components)
- simulate photo bleaching
- simulate cell cycle

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