1. Within the field of quantitative microscopy the thesis "good image processing starts with good image acquisition" can be extended to "good image processing starts with good sample preparation". (*this thesis*)

2. Due to the problems in achieving correct Köhler illumination in fluorescence microscopy it is recommended to move the mirror image of the arc lamp completely out of the illumination field.

3. The image intensity in epi-fluorescence microscopy is in general independent of the magnification of the objective lens. (*this thesis*)

4. It is not necessary to acquire the nucleus image and FISH probe image separately with automated dot counting of single hybridized specimens. (*this thesis*)

5. The large amount of information on the internet makes it necessary to develop new search methods, since the present methods are either too selective or not selective enough.

6. Given public opinion, the Dutch phrase for genetic engineering "genetic manipulation" should be changed to "genetic modification".

7. Soccer, it's just a game.

8. Given the large number of Ph.D. graduations each year and the limited funds of the university, the graduation procedure should be simplified.

9. Image processing software without a camera interface is like a word processor without a printer. You can practice whatever you want, but you will never be productive.

10. For the encouragement of vegetarianism, the cow disease BSE is not so "mad".
Stellingen

Behorende bij het proefschrift

Automated Image Analysis of FISH-Stained Cell Nuclei

Hans Netten
11 maart 1997

1. De stelling "goede beeldverwerking begint met een goede beeldopname" kan binnen de kwantitatieve microscopie worden uitgebreid tot "goede beeldverwerking begint met een goed sample preparaat". (dit proefschrift)

2. Vanwege de problemen bij het juist instellen van Köhler belichting in fluorescentie microscopie verdient het aanbeveling het spiegelbeeld van de lamp volledig uit het belichtingsveld te draaien.

3. Bij fluorescentie microscopie met opvallende belichting is de beeldintensiteit in het algemeen onafhankelijk van de vergroting van het objectief. (dit proefschrift)

4. Bij het automatisch spot tellen van enkelvoudige gehybridiseerd preparaten is het niet nodig om sequentieel het kern en FISH probe beeld apart op te nemen. (dit proefschrift)

5. De grote hoeveelheid informatie op het internet maakt het noodzakelijk dat er andere zoekmethoden worden ontwikkeld dan de huidige methoden, aangezien deze meestal te selectief zijn dan wel niet selectief zijn.

6. Wat betreft de publieke opinie zou genetische manipulatie beter genetische modificatie kunnen worden genoemd.

7. Voetbal, het is maar een spelletje.

8. Gegeven het grote aantal promoties per jaar en de beperkte financiële middelen van de universiteit zou het zinvol zijn de protocollaire procedure rondom een promotie sterk te vereenvoudigen.

9. Software voor beeldverwerking zonder camera interface is als een tekstverwerker zonder printer. Je kunt er leuk mee oefenen maar produktief word je er niet mee.

10. Ter bevordering van het vegetarisme is de koeienziekte BSE nog niet zo gek.
Automated Image Analysis of FISH-Stained Cell Nuclei
Automated Image Analysis of FISH-Stained Cell Nuclei

PROEFSCHRIFT

ter verkrijging van de graad van doctor
aan de Technische Universiteit Delft,
op gezag van de Rector Magnificus Prof.dr.ir. J. Blaauwendraad,
in het openbaar te verdedigen ten overstaan van een commissie,
door het College van Dekanen aangewezen,
op dinsdag 11 maart 1997 te 16.00 uur

door

Hans NETTEN

natuurkundig ingenieur
geboren te Son en Breugel
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Prof.dr. I.T. Young
Prof.dr. H.J. Tanke

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Aan Yvonne
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Chapter 1

General Introduction

This chapter provides some background information on fluorescence in situ hybridization and the relation with digital image analysis. Biological aspects, such as DNA, chromosomes, mutations and the cell cycle are briefly discussed. Modern molecular technology has made it possible to selectively stain various DNA sequences in biological cells. This technique is called fluorescence in situ hybridization (FISH) and is nowadays one of the most promising procedures in cytogenetics. The motivation to use digital image analysis with FISH applications is discussed. Finally an overview of this thesis is given.
Fluorescence microscopy is an essential tool for the examination of cells and cellular constituents. The development of fluorescent dyes and cytochemical labeling techniques have lead to an increased interest in fluorescence microscopy. Attaching fluorescent dyes to specific molecular cell constituents allows the visualization and quantization of the biological reaction of these constituents in morphologically well-preserved cells or tissue. Modern molecular technology has made it possible to selectively stain various DNA sequences in biological cells. These sequences may be chosen so as to detect specific abnormalities or to facilitate the process of counting chromosome types. This technique is based on the interaction of complementary nucleic acid sequences and is called fluorescence in situ hybridization (FISH). FISH is nowadays one of the most promising procedures in cytogenetics. Applications of in situ hybridization techniques involve, among others, tumor cytogenetics, prenatal diagnosis and gene mapping. Digital imaging technology plays an important role in the development of FISH applications. It enables the visualization of the weak fluorescent signal, quantitative information can be obtained, and certain tasks can be automated.

The instrumentation that is necessary for the analysis of FISH preparation is the main topic of this thesis. We have focused on one particular application, chromosome enumeration. This chapter provides some background information on fluorescence in situ hybridization and the relation with digital image analysis. Biological aspects and FISH methodology are briefly discussed. The motivation to use digital image analysis with FISH applications is discussed in section 1.3. The last section gives an overview of this thesis.

1.1 Biological background

Living cells contain a macro molecule that is called DNA (Deoxyribonucleic Acid). The DNA contains all the genetic information that is responsible for cell differentiation, cell growth, and the functionality of a particular cell. The total amount of DNA within the cell is called the genome. All cells of one organism originate from a single cell through cell division. Before a cell divides into two new cells, the genome is duplicated and thus each cell will contain the same genetic information.

At the molecular level a DNA molecule is composed of two strands of a deoxyribose-phosphate backbone. The two strands are connected by a sequence of base pairs. The DNA is double-stranded and has a geometric structure of a double helix. There are four different bases: adenine (A), guanine (G), cytosine (C), and thymine (T) and they can form two base pairs, C-G and A-T. Cytosine is always connected to guanine and adenine is always connected to thymine and vice versa. This means that the two strands are exact
Complements. The genetic information is determined by the specific sequences of these base pairs along the chain. The genome of a human cell contains about $3 \times 10^9$ base pairs. These base pairs are grouped into about 100,000 genes, where each gene contains the instructions for the building of a specific protein. The human genome is divided into 46 parts, called the chromosomes. There are 22 pairs of different chromosomes, called autosomes. Autosomes are numbered from 1 to 22 where chromosome 1 is the largest autosome. The two remaining chromosomes are the sex chromosomes. The sex chromosomes of a female are a pair of X chromosomes and the sex chromosomes of a male are one X and one Y chromosome. The individual chromosomes can be visualized with microscopic techniques (see section 1.2) during the cell division phase of the cell cycle.

Cell division is central to life in all organisms. In this process the DNA is duplicated and divided among the two newly created cells. This type of cell division is called mitosis. The process of cell growth and division is called the cell cycle. The cell division can be divided into four different stages, prophase, metaphase, anaphase, and telophase. The remaining time is called interphase. During interphase the DNA is contained in a more or less spherical compartment, the nucleus. The interphase can also be divided into three parts, the G1-gap, the S-phase, and G2-phase. The G1-gap is the period between the end of the cell division and the beginning of the DNA replication. During S-phase DNA is synthesized and the chromosomes are duplicated, such that the nucleus contains twice the amount of DNA in the G2-phase. After the G2-phase the actual cell division starts with prophase. The chromatin (DNA combined with packing proteins called histones) condenses into chromosomes. Each chromosome consists of two sister chromatids joined at a specific point along their length, called centromere. When condensation has been completed and the nuclear membrane has disappeared, all chromosomes are positioned such that their centromeres all lie in one plane (metaphase). During the subsequent anaphase, the two chromatids are separated and moved to separated areas. Finally the condensed chromosomes expand and nuclear membranes reappear after which two new cell are formed (telophase).

Due to the complex process of the cell cycle, all kinds of errors may occur during the DNA replication. Because the DNA regulates the behavior of a cell, a disorder in the sequence of base pairs can have severe consequences. When a change occurs in the sequence it is called a mutation. Mutations occur naturally as a consequence of imperfectness of the replication mechanism or can be caused by the influence of radiation, toxic agents, and viruses. The consequences of a mutation depend largely on the cell type, the place of the mutation, and the kind of mutation. Various types of mutations can be distinguished: point mutation (a single base pair is replaced), deletions (a number of base pairs is lost), insertions (a number of base pairs is inserted),
translocation (an exchange of base pairs between two chromosomes), and inversion (a part of a chromosome is in a reverse position). Not every mutation will have serious consequences because only a part of the genes are is for one particular cell and not all the DNA is used for the genetic information. The majority of the DNA (more than 90%) consists of non-coding parts. But if a mutation occurs in a gene which regulates the cell division, for example, this can lead to a cancerous growth of tumors. Besides structural aberrations also numerical aberrations may occur. When this results in the loss of one autosome this is called monosomy and when an extra copy is produced, it is called trisomy.

![Metaphase Image](image)

**Figure 1.1:** Giemsa stained metaphase and the resulting karyotype.
1.2 Fluorescence in situ hybridization (FISH)

To detect and analyze chromosomal aberrations various techniques have been developed in the last 50 years. A well known technique is the chromosome banding method. This staining technique results in an intensity banding pattern along the length axis of the chromosomes. It allows the characterization of individual chromosomes and the detection of certain regions or bands on the chromosome. The ordering of the individual chromosomes, based on their size and banding pattern, is called karyotyping. This technique can only be applied on metaphase cells. The smallest band that can be detected is on the order of a few million base pairs. Figure 1.1 shows a microscopic image of a Giemsa stained metaphase and the resulting karyotype. The images are screen snapshots of a semi-automatic karyotyping system, Athena (Van Vliet et al 1990).

![DNA sequence](image)

**Figure 1.2**: A schematic diagram of the *in situ* hybridization process. The DNA sequence is first separated by denaturation and then hybridized with a labeled probe.

Modern molecular technology has made it possible to selectively stain various DNA sequences on biological cells, which can not be detected using the described banding technique. *In situ* hybridization (ISH) allows the visualization of specific DNA targets in a morphologically well-preserved metaphase or interphase cell. This method is based on the fact that under appropriate conditions, single stranded DNA can form duplexes with complementary strands. This process is called hybridization. *In situ* hybridization
(application of the technique to individual cells and chromosomes) is a method where the double helix DNA of a cell is first separated by denaturation and then hybridized with a labeled probe to localize the DNA sequence of interest (see figure 1.2). The probe is also a sequence of single-stranded DNA and is the complement of the target sequence. Depending on the type of the probe, one can label a unique DNA sequence of a chromosome, a region of a chromosome (for example the centromere), or even the whole chromosome. Figure 1.3 shows a typical FISH image of a single hybridized metaphase cell and interphase cell. A centromeric probe is used to label the centromere of chromosome 12 and the centromeres appear as bright dots in the image. The ability to analyze interphase nuclei has the advantage that it can be applied to preparations containing few or poor metaphase spreads and to solid tumor tissue (Vrolijk 1993). This is especially important for applications where a large number of cells has to be analyzed.

Figure 1.3: A typical FISH image of a double stained metaphase cell and interphase cell. A centromeric probe has been used to label chromosome 12.

Probes were originally labeled with radio-isotopes. The disadvantage of this labeling technique is that it has a poor spatial resolution because of the tracks of the decaying particles in the silver emulsion, multiple targets are difficult to distinguish, and there is a health hazard associated with handling radioactive material. Nowadays the visualization is achieved with labels based on fluorescence or absorption. For relatively simple cases it is possible to apply probes based on light absorbing dyes (Vrolijk et al 1996). However, fluorescence in situ hybridization (FISH) is the most exciting and promising procedure.
When excited by light of certain wavelengths, fluorescent molecules emit light at longer wavelengths. Using a fluorescence microscope and a proper filter block the fluorescent dye becomes visible as colored regions. Fluorescence has a high sensitivity and allows the detection of small targets. Multiple targets can be visualized simultaneously by using different dyes with different colors. And, in addition, fluorescence allows the quantization of the target molecules, since theoretically the fluorescence intensity is a monotonic increasing function of the concentration of the fluorescent molecules (under appropriated conditions).

FISH techniques have significant potential both in research and in clinical applications. The probes may be chosen so as to detect specific abnormalities or to facilitate the process of identification and quantification of numerical and structural chromosomal abnormalities (Eastmond and Pinkel 1989, Hopman et al 1991, Nederlof et al 1989). By labeling human chromosome 21, for example, it becomes possible to identify three chromosomes (trisomy) instead of the normal two chromosomes. This particular aberration is associated with Down's syndrome.

Compared to the classical chromosome banding methods, FISH has several advantages. Using banding techniques only relatively large aberrations, on the order of a few million base pairs, can be detected. FISH has a high sensitivity as sequences of a few hundred base pairs can be localized in metaphase chromosomes. Other drawbacks of chromosome banding methods are that the interpretation of the karyotypes is time consuming, requires highly experienced personnel and results are frequently limited by samples with nonclonal abnormalities, poor availability of dividing tumor cells and poor metaphase quality. FISH allows the same information to be obtained from non-dividing cells as well as dividing cells, it is less affected by metaphase quality, and results are often easier to interpret requiring less training and experience. The latter gives the opportunity to automate the analysis of FISH specimens. Automation in combination with the availability of specific cytogenetic markers makes it possible to rapidly analyze large number of cells. The ability to analyze interphase cell nuclei is called interphase cytogenetics (Cremer et al 1986) and has the advantage that it can be applied to preparations containing few or poor metaphase spreads and to solid tumor tissue. The latter case interphase cytogenetics is helpful since it circumvents problems of culturing tumor cells and in addition the architectural information of the tissue can be maintained.

Different clinical applications have been reported such as the detection of minimal residual disease, early relapse detection in leukemia's, prenatal diagnosis, and tumor analysis (Arnoldus et al 1989, Perez Losada et al 1991, Ward et al 1993). A typical example of an application of FISH for cytogenetic diagnostics has been reported by Kibbelaar (Kibbelaar et al 1993). They have compared conventional banding techniques and FISH for the detection of monosomy 7 and trisomy 8 in 89 patients with myeloid
malignancies. Monosomy 7 and trisomy 8 are the most common numerical chromosome aberrations in myelodysplastic syndromes and acute myeloid leukemia. They have shown the feasibility and potential of FISH analysis for this particular application.

Although there is large diversity of FISH applications, the analysis of the specimens shows many similarities. The analysis of a FISH specimen consists in general of detecting the probe signals after which the number of signals can be counted, the intensity can be measured, and/or their relative positions can be determined. Chromosome enumeration becomes a matter of detecting the FISH signal in interphase cells and then counting the number of signals, assuming that the probe reflects the presence of a chromosome. In case it is a centromeric specific probe this will not always be the case. Gene mapping again requires the detection of the different probe signals after which their relative position can be measured (Lichter et al 1990). Ratio labeling requires an accurate measurement of the fluorescence intensity of the different dyes to distinguish the different probes based on their color ratio (Nederlof et al 1992 b).

1.3 Digital Image analysis

The possibilities of FISH methods are considerably increased by the use of modern digital imaging technology. Modern detection devices such as charge coupled device (CCD) cameras allow the detection and visualization of fluorescence signals that can not be detected by the human eye. Quantitative data can be obtained and certain tasks can be automated using image processing and image analysis techniques. The applications can be divided into those in which the goal is the production of images that are to be used as images by human observers and those where the images are analyzed to produce data for human interpretation (Young 1996). The process of "specimen in" → "image out" is called image processing. If we can speak of "specimen in" → "data out" this is called image analysis. The motivation to use digital imaging techniques with FISH applications can be divided into three categories:

• Visualization — The consequence of the weak fluorescent signal is that small targets are hardly visible when one is looking through the eyepieces of a fluorescent microscope. CCD cameras have the capability to integrate for several seconds. If enough photons have been accumulated, the image can be displayed on a computer monitor. Image processing techniques like restoration and image enhancement can improve the visualization of the FISH images.

• Quantitative analysis — A number of FISH applications are based on the measurement of features that cannot be obtained manually. While qualitative measurements answer the
question about the presence or absence of an object of interest, a quantitative measurement gives the opportunity to give a quantitative description of the biological process. Digital measurement theory provides the user with a number of tools to measure size, shape, and intensity features accurately.

- Automation — The large amount of work that is involved with the analysis of FISH specimens of some application may require that certain tasks are automated. FISH has great potential for clinical applications. In a clinical situation the number of specimens that have to be analyzed can be large. Some applications are based on statistics and if the number of aberrant cells is low, the number of cell that have to be analyzed can be very large (Carothers 1994, Castleman and White 1995).

In practice it will be a combination of these three aspect that motivates the use of digital image analysis. A complete system for automated analysis of FISH images consists of an automated fluorescence microscope, a camera to digitize the images, and a computer that analyzes the images and controls the whole process. Depending on the application, the software must consist of an auto-focusing algorithm, a scanning algorithm, and specific image processing algorithms to obtain the required results. Finally the system must be provided with a user-interface to control the microscope, to visualize the images, and to verify and correct the results.

1.4 Scope of this thesis

In principle all aspects of digital image analysis system for FISH specimens will be discussed in this thesis. Such a system contains all of the components common to image processing and image analysis: image acquisition, auto-focusing, automated scanning, segmentation, measurement, and classification. The large diversity of topics does not permit us to discuss every aspect in detail. Although most of the topics will be discussed in a more or less general way we have focused on one particular application, chromosome enumeration.

Chromosome enumeration is commonly called dot counting. FISH signals in interphase cells become visible as colored dots. The analysis of a preparation consists of detecting the dots after which the number of dots can be determined. With chromosome enumeration, dots are counted for a large number of cells to determine the distribution of chromosomes per cell and to be able to detect small aberrant sub-populations. The number of cells that have to be analyzed depends on the frequency of aberrant cells, the reliability of the FISH procedure and the count accuracy. In practical situations this can vary from only a few cells to more then 10,000 cells (Carothers 1994, Castleman and
Current manual counting procedures leave much to be desired including the need to work in a darkened environment and the fatiguing nature of the work. If the required number of analyzed cells is too large, manual counting will not be possible in practice, because it would require too much time. An automated dot counting system is required.

This thesis describes the development of a completely automated microscope system that counts fluorescent hybridization dots for one probe in interphase cell nuclei. Only two colors, such as DAPI and Spectrum Orange™, can be used - one for the counter stain and one to make the chromosome visible. The aim of this project was to develop a system that is comparable to manual dot counting. Manual obtained results have been used as "ground truth". The required speed of the system is on the order of 500 nuclei per 15 min. which is at least comparable to manual counting. The automated dot counter has been tested on a number of slides where DAPI was used for the nucleus counter stain and a centromeric 8 or 12 probe (CEP 8 and CEP 12, Vysis, Downers Grove, IL, USA) was used to mark the desired chromosome. The probe was labeled with Spectrum Orange. The slides contained lymphocytes from cultured blood. All slides were from normal specimens. The samples have been provided by Vysis and the Department of Cytochemistry and Cytometry (University of Leiden, Leiden, The Netherlands). Most of the experiments have been done with the samples from Vysis. Where other samples are used, this will be mentioned.

This thesis is organized as follows. Chapter 2 describes the image acquisition procedure. This is the process that starts with a certain specimen and results in the digitized image stored in computer memory. The different components, such as fluorescence microscope, camera, and scanning algorithm will be discussed and characterized. All these components have to be configured is such a way that the performance will meet the required image quality and speed. Chapter 3 describes the design of a special image acquisition system for fluorescence microscopy using a 2-D CCD and time delayed integration (TDI). This chapter was originally published in Bioimaging (Netten et al 1994). Instead of a start-stop system we use continuous stage motion in the CCD's parallel shift direction. Synchronizing the parallel clock and the stage velocity guarantees a one-to-one relationship between a moving cell and its image onto the CCD. Chapter 4 gives a complete description of the image analysis procedure of the dot counter. The procedure consists of finding nuclei in the image, detecting the dots within the nuclei, counting the number of dots per nucleus and updating the results including a dot histogram for the entire specimen. We will focus on the dot detection algorithm. Three different algorithms are described. The problem of "overlapping" dots and split dots is

---

(TM) Spectrum Orange is a trademark of Vysis Corporation, Downers Grove, Illinois, USA
discussed. The automated dot counter has been tested on a number of normal specimen. The performance of the different algorithms has been evaluated and compared with manual counting. The experimental results are given in chapter 5. An error analysis is given and different error causes are discussed. The work presented in chapter 4 and 5 was originally published in Bioimaging and Cytometry (Netten et al 1996 a, Netten et al 1996 b). Finally, in chapter 6, we draw some general conclusions and the results are discussed.
The automated analysis of FISH specimens consists of an image acquisition procedure, followed by an image analysis procedure. The image acquisition procedure covers the whole process that starts with a certain specimen and results in the digitized images stored in computer memory. In fluorescence microscopy we have to deal with low light intensities of the fluorescent dyes. Different dyes can be distinguished based on their color. Color information must be obtained. This chapter describes the whole image acquisition procedure. The goal of this chapter is to get a better insight into the performance of the image acquisition system and how the different parameters can be chosen based on the requirements of an application. A complete automated system consists of a fluorescence microscope, motor driven stage, focus and filter control, camera, frame grabber and a computer that controls the entire process. All these components have to be configured in such a way that the performance of the system will meet the required image quality and speed. Imaging strategy, scanning method and auto-focusing procedure will be characterized and discussed.
2.1 Introduction

The automated analysis of FISH specimens can be divided in two parts: 1) the image acquisition, and 2) the image analysis. The image acquisition procedure covers the whole process that starts with a certain specimen and results in the digitized images stored in computer memory. The use of such a microscope system for the automated analysis of FISH specimens requires an understanding of: the fluorescence process, the characteristics of microscope optics and the characteristics of the imaging device. A complete automated system consists of a fluorescence microscope, motor driven stage, focus and filter control, camera, frame grabber and a computer that controls the entire process. All these components have to be configured in such a way that the performance of the system will meet the required image quality and speed. The required image quality depends on the application and will be expressed in terms of the resolution, depth-of-focus (DOF), contrast, and signal-to-noise ratio (SNR). Detecting fluorescent dots e.g. could require a high contrast in combination with a large depth-of-focus. In the case of measuring the intensity ratio between two dyes, the resolution and SNR would be important features.

Nowadays, different microscope systems and cameras are available. Different configurations can be developed. Besides the type of microscope or camera, important parameters like numerical aperture, sampling density and integration time have to be chosen. The goal of this chapter is to get a better insight into the performance of the image acquisition system and how the different parameters can be chosen based on the requirements of an application. The fluorescence microscope and imaging device will be discussed. The different components will be characterized and evaluated. The last section describes how the actual screening is implemented. The imaging strategy, scanning method and auto-focusing will be discussed. The application of automated FISH dot counting in interphase cell nuclei is used as an example to illustrate the different topics. But the work presented can be applied to other FISH applications.

2.2 Fluorescence microscope

The fluorescence microscope is an essential tool for the visualization of FISH specimens. Computer controlled scanning requires the automation of stage, focus, and filter wheels. This section describes and characterizes the basic building blocks of a fluorescence microscope.
2.2.1 System overview

A schematic overview of an automated fluorescence microscope system is given in figure 2.1. The whole system is built around an epi-illuminated fluorescence microscope. Focus, stage, filter wheels, and excitation shutter are controlled by a computer. The microscope images are acquired using a camera that is mounted (in our case) on top of the microscope. We have used two different configurations for the development of the automated dot counter. One system is based on a Leica Aristoplan (Ernst Leitz Wetzlar GmbH, Germany) and the other is based on a Zeiss Axioskop (Carl Zeiss, Oberkochen, Germany).
Table 2.1: Some specifications of the two microscope systems that are used in the development of the automated dot counter. One system is based on a Zeiss Axioskop microscope and the other is based on a Leitz Aristoplan microscope. The speed of the stage is defined as the time to move from one field to the next field, the focus speed is defined as the time to move 1 μm, and the speed of the filter wheel is defined as the average time to switch between two filters.

<table>
<thead>
<tr>
<th>System specifications</th>
<th>Zeiss system</th>
<th>Leica system</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Microscope</strong></td>
<td><em>Axioskop</em></td>
<td><em>Aristoplan</em></td>
</tr>
<tr>
<td>Illumination source</td>
<td>Mercury (100 W)</td>
<td>Mercury (100 W)</td>
</tr>
<tr>
<td>Optics</td>
<td>Infinity color-corrected (ICS)</td>
<td>-</td>
</tr>
<tr>
<td>Objectives</td>
<td>x25/0.8 Plan Neofluar, multi im.</td>
<td>x40/1.3 Plan Fluotar, oil im.</td>
</tr>
<tr>
<td></td>
<td>x40/1.3 Plan Neofluar, oil im.</td>
<td>x25/0.6 Plan Fluotar</td>
</tr>
<tr>
<td>Relay lens</td>
<td>x1</td>
<td>x1, x1.25, x1.6, x2</td>
</tr>
<tr>
<td>Camera port</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td><strong>Hardware control</strong></td>
<td><em>Ludl - Mac 2000</em></td>
<td><em>Märzhäuser - Mac 4000</em></td>
</tr>
<tr>
<td>Interface</td>
<td>1 serial line (RS 232)</td>
<td>2 serial lines (RS 232)</td>
</tr>
<tr>
<td><strong>Stage</strong></td>
<td><em>Ludl</em></td>
<td><em>Märzhäuser</em></td>
</tr>
<tr>
<td>Resolution (nm/step)</td>
<td>100</td>
<td>50</td>
</tr>
<tr>
<td>Speed (msec)</td>
<td>400</td>
<td>260</td>
</tr>
<tr>
<td><strong>Focus</strong></td>
<td><em>Ludl</em></td>
<td><em>Märzhäuser</em></td>
</tr>
<tr>
<td>Resolution (nm/step)</td>
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<td>1</td>
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<tr>
<td>Speed (msec)</td>
<td>350</td>
<td>140</td>
</tr>
<tr>
<td><strong>Excitation filter wheel</strong></td>
<td><em>Ludl</em></td>
<td><em>Leica</em></td>
</tr>
<tr>
<td>Filters</td>
<td>5</td>
<td>4 + 1 closed</td>
</tr>
<tr>
<td>Speed (msec)</td>
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<td>420</td>
</tr>
<tr>
<td><strong>Emission filter wheel</strong></td>
<td>-</td>
<td><em>Leica</em></td>
</tr>
<tr>
<td>Filters</td>
<td>-</td>
<td>5</td>
</tr>
<tr>
<td><strong>Excitation shutter</strong></td>
<td><em>Ludl</em></td>
<td>-</td>
</tr>
</tbody>
</table>

Both microscopes can be used for bright-field microscopy as well as fluorescence microscopy. Bright-field microscopy is not within the scope of this thesis. Table 2.1 gives the specifications of the two configurations. The functionality is almost the same for both systems. The Aristoplan does not have an excitation shutter. The excitation filter wheel is used to block the light. The Aristoplan has two camera ports with a selectable relay lens and an emission filter wheel that makes the system more flexible. All hardware components are driven by stepping motors. Some features of the stage, focus control, and filter wheel are given. The speed of a stage has been measured as the time to move the stage from one field of view (FOV) to the next FOV using a Photometrics KAF 1400 camera (see section 2.3) in combination with a x40 magnification. The speed of the focus...
device has been defined as the time to move 1.0 μm, which is on the order of the focus step size. Finally, the speed of the filter wheel is the average time to switch between two filters. These numbers show that there is not much difference between the Ludl (Ludl Electronic products Ltd.) and Märzhäuser (Märzhäuser Wetzlar, Germany) hardware.

![Figure 2.2](image.png)

**Figure 2.2:** The illumination path of an epi-illuminated fluorescence microscope. The filter block selects the proper illumination spectrum, illuminates the specimen via the chromatic beam splitter, and selects again the proper emission spectrum.

### 2.2.2 Illumination

Figure 2.2 illustrates a typical light path in an epi-illuminated fluorescence microscope. A mercury 100 W lamp is used to illuminate the specimens. The filter block consists of an excitation filter, a chromatic beam splitter, and an emission filter. The excitation filter selects the proper wavelengths that are suitable for the fluorescent dye to illuminate the specimen via the chromatic beam splitter. The chromatic beam splitter reflects the excitation wavelengths while it passes the emission wavelengths. The emission filter finally selects the proper wavelengths of the emission spectrum of the dye. The excitation and emission filter are band-pass filters and interference filters are commonly used. Often multiple targets have to be visualized and therefore different dyes are used simultaneously. In that case, a multiple band-pass filter block can be used. A dual band-pass filter block excites two dyes simultaneously and passes the emitted light of the two dyes. We have used both a dual bandpass excitation filter and the equivalent single excitation filters, in combination with a dual bandpass emission filter (Omega Optical Inc., Brattleboro, USA), that are suitable for DAPI and Spectrum Orange. The spectral characteristic of the dual band-pass emission filter is given in figure 2.3. The maxima of the emission spectra of DAPI and Spectrum Orange are at λ = 461 nm and λ = 615 nm, respectively.
Figure 2.3: The relative transmission of a dual band-pass emission filter suitable for DAPI and Spectrum Orange.

2.2.3 Microscope objectives

The compound microscope is a combination of two optical systems, the objective and ocular. The most important component in the optical path of the epi-fluorescence microscope is the objective, since it is used as condenser and as objective lens. The quality of the acquired images, in terms of resolution, depth-of-focus, contrast, and brightness, depends on the performance of the objectives. Objective lens manufacturers provide the user with a whole range of objective with different magnifications, numerical apertures, and special features. Objectives could be corrected for spherical aberration, chromatic aberration, astigmatism, and coma. A more detailed discussion of objective aberrations can be found in (Inoué 1986). High quality microscope images are achieved with plan-apochromatic objective lenses. For low-light-level imaging, in particular fluorescence, the so called Fluor objectives are commonly used. The auto-fluorescence of "normal" objective can be large, specially in the UV range. The special glass used in the Fluor objectives reduces the auto-fluorescence.

Besides the different features of the objectives there are two important parameters, the magnification ($M_{obj}$) and the numerical aperture (NA). Brightness, resolution, and depth-of-focus are related to these two parameters.

- Brightness — The image brightness is defined as the average light intensity per unit area at the sensor plane and is equal to the average measured pixel intensity $I(x,y)$. The image brightness at the sensor plane of an epi-fluorescence microscope is commonly assumed to be proportional to the fourth power of the NA and inversely proportional to the square of the overall magnification $M_{obj}M_{rel}$ (Inoué 1986), and is given by:
where \( M_{obj} \) is the magnification of the objective lens and \( M_{rel} \) is the magnification of the relay lens. Equation 2.1 is correct if there is Köhler illumination and the image of the light source is larger than the condenser aperture. Experimental results (Piper 1996), showed that the measured data did not agree with the prediction of eq. 2.1. It showed that the measured brightness with a constant relay magnification is more closely proportional to \( NA^2 \) and independent of the objective magnification. Piper explained these results as a consequence of the small size of the mercury arc. The light source is a point rather than an extended uniform source. The image of the source at the condenser back aperture is also a point and thus will not fill the aperture.

\[
I(x,y) \propto \frac{NA^4}{M_{obj}^2 \cdot M_{rel}^2}
\]  

(2.1)

**Figure 2.4**: Köhler illumination of an epi-fluorescence microscope. In this example the light source fills the condenser aperture. The brightness at the object plane is then proportional to \( NA^2 \) and is independent of the magnification.

The reasoning that leads to eq. 2.1 is as follows. The brightness at the sensor plane depends on the light-gathering power of the objective lens and on the illumination brightness of the excitation source at the object plane. The fluorescence intensity of the dyes is under proper conditions proportional to the illumination intensity. Essential in epi-
fluorescence microscopy is that the objective lens is used for the image formation as well for the illumination of the objects. In other words, if the objective lens is changed to a different magnification and/or numerical aperture, the magnification and/or numerical aperture of the condenser are changed as well.

Assuming that the image of the light source is larger than the condenser aperture (see figure 2.4) and the source luminance is uniform across the aperture, the brightness at the object plane is proportional to $NA^2$ and is independent of the magnification. The reason is that under Köhler illumination, light emanating from each point on the image of the light source at the condenser aperture uniformly illuminates the microscope field. Therefore the brightness at the object plane is proportional to the area of the condenser aperture. The area of the condenser aperture $A_{con}$ is proportional to $NA^2/M^2_{obj}$ (Inoué 1986). The brightness is also inversely proportional to the area of the illumination field $A_{ex}$. The area of the field that is illuminated is inversely proportional to $M^2_{obj}$ without considering the field stop. The size of the opening in the field diaphragm affects only the diameter of the field that is actually illuminated and not its brightness. Combining these two results, the resulting brightness at the object plane is proportional to $NA^2$ and the magnification is canceled out. The light-gathering power of the objective lens is proportional to $NA^2/M^2_{obj}$ (Inoué 1986). Combining the illumination and image forming aspects together and taking the relay magnification into account the expected brightness at the sensor plane is given in eq. 2.1.

But in the case that the light source is small, the image of the source will not completely fill the condenser back aperture. The consequence is that the illumination brightness is independent of the area of the condenser aperture and therefore not proportional to $NA^2/M^2_{obj}$. A smaller condenser aperture does not reduce the amount of light that passes through the lens. The illumination brightness at the object plane is still proportional to $M^2_{obj}$. The image formation is the same as before which results in a light-gathering power that is proportional to $NA^2/M^2_{obj}$. Combining these results, the brightness at the sensor plane is given by:

$$I(x,y) \propto \frac{NA^2}{M^2_{rel}}$$  \hspace{1cm} (2.2)

We have used with both microscope systems the HBO 100 W arc lamp that has an arc diameter of about 0.25 mm (Inoué 1986). Because the magnification of the illumination optical train is not known for both microscope systems, the size of the arc is measured at the condenser aperture. The diameter of the arc image is approximately 3 mm. The radius of the opening of the objectives is also measured with an ordinary ruler. For all objectives that have been used the pupil size of the lens is larger than the 3 mm image of the arc. To verify the results of eq. 2.2 we have measured the average intensity of a homogenous FOV with 5 different objectives. The results have been obtained using the Leica microscope system and the Sony camera (see section 2.3). Figure 2.5 shows the average
pixel intensity as a function of $NA^2$. The test slide contained a solution of TRITC (maximum emission spectrum at $\lambda = 578$ nm). The air bubbles in the solution are used for focusing. It can be seen that the measured brightness is closely proportional to $NA^2$. The coefficient of regression is $R^2 = 0.96$. The correspondence with the prediction of eq. 2.1 is not very close ($R^2 = 0.78$). Our results are in agreement with the results from Piper. Although the brightness is independent of $M_{obj}$, it is still inversely proportional to the square of the relay magnification $M_{rel}$.

![Normalized average intensity vs. $NA^2$](image)

**Figure 2.5:** The average intensity as function of $NA^2$. The following objectives have been used: x16/0.45, x25/0.6, x40/1.3 (oil im.), x50/1.0 (water im.), and x63/1.4 (oil im.). The test slide contained a solution of FITC. A linear fit of the data results in a coefficient of regression $R^2 = 0.96$.

- Resolution — The resolution is a measure of the smallest detail that can still be resolved. There are several ways to define the resolution of an optical system. Often the Rayleigh criterion is used to define the resolution. The Rayleigh criterion defines a distance at which two superimposed Bessel distributions can still be separated based on the intensity. Assuming Köhler illumination, the optical system of a fluorescence microscope can be described as a linear shift invariant (LSI) system (Born and Wolf 1959, Williams and Becklund 1989). Linear system theory can then be used to describe the optical system. An infinite small point source, within the focal plane, will be observed as an Airy disk which is the square of the Bessel function mentioned above if the lens is
diffraction limited. This is also called the point spread function (PSF) of the optical system. The Rayleigh distance is given by:

$$d = 0.61 \cdot \frac{\lambda}{NA}$$  \hspace{1cm} (2.3)

where $\lambda$ is the wavelength of the emitted light. The distance is equal to the position of the first dark ring of the Airy disk relative to the center of the disk. If the $NA$ increases the smallest resolvable distance between two points decreases. The disadvantage of the Rayleigh criterion as a measure of the resolution is that it is based on the visual examination of an image. Instead of describing the performance of an objective in term of the PSF, we can also consider the spatial frequency response of the objective. The Fourier transform of the PSF is called the optical frequency transfer function (OTF). Figure 2.6 shows an example of the PSF and the corresponding OTF. It is important to realize that the optical system is a band-limited system. The OTF is zero above the cut-off frequency $f_c$. The cut-off frequency is given by:

$$f_c = 2 \cdot \frac{NA}{\lambda}$$  \hspace{1cm} (2.4)

The cut-off frequency is inversely proportional to the Rayleigh distance. A high $NA$ objective will have a narrow PSF and a wide OTF.

![Figure 2.6: The theoretical point spread function of a microscope system with the corresponding Fourier transform, the optical transfer function.](image-url)

- **Depth-of-focus** — The depth-of-focus is defined as the distance $\Delta z$ over which the microscope image can be expected to be observed without significant optical aberration. The problem with this definition is that a criterion has to be chosen to define what is significant. The depth-of-focus has been describe by a number of authors. Unfortunately,
these descriptions are not applicable in our case. Two of the most well-known formulas are from (Piller 1977) and (Born and Wolf 1959). Piller proposed that $\Delta z$ is a function of $\lambda$, $NA$, and $M$ and is given by:

$$\Delta z = \frac{1000}{7 \cdot NA \cdot M} + \frac{\lambda}{2 \cdot NA^2}$$  \hspace{1cm} (2.5)$$

The formula is suitable for objectives that have large aberrations. Born and Wolf proposed a formula that is only suitable for low NA objective. Using wave theory another formula has been derived (Longhurst 1967, Young et al 1993):

$$\Delta z = \frac{\lambda}{4 \cdot n \cdot \left(1 - \sqrt{1 - (NA/n)^2}\right)}$$  \hspace{1cm} (2.6)$$

This formula is based on the criterion that the maximum wavefront aberration due to an axial shift does not exceed one fourth of the wavelength $\lambda$. This corresponds with the Rayleigh criterion that says that two wavefronts can be distinguished if their path difference is larger then $\lambda/4$. Eq. 2.6 shows that the depth-of-focus is a function of the numerical aperture $NA$, the index of refraction $n$, and the wavelength $\lambda$, but not a function of the magnification $M$. Using the typical values of $\lambda$=615 nm, $NA = 1.3$, and $n$=1.5, the depth of focus becomes $\Delta z = 0.2 \ \mu m$. The depth-of-focus will be small if high $NA$ objectives are used.

The question if eq. 2.6 is a good measure for the depth-of-focus when it is used with quantitative image analysis still remains. For example, if we want to measure the area of a relatively large object, the error, due to an axial shift, will be small within a range that is much larger than the depth-of-focus as defined by eq. 2.6 (Ellenberger and Young 1996). If the objects are smaller, the influence of the focus position becomes larger. This example shows that the tolerance of the focus position is not only determined by the depth-of-focus of the optical system but also by the application. FISH permits the labeling of small regions of a chromosome. The probes appear as bright dots in the image. The size of the dots is generally on the order of the size of the PSF. The PSF is a function of focus position. Dots that are out-of-focus will appear larger and with less contrast then when they are in focus.

This is illustrated in figure 2.7. A sequence of images has been acquired at different $z$ positions of a fluorescent bead (Cat. No. L-5249, Molecular Probes, Inc., Eugene, OR, USA) with a diameter $d = 0.2 \ \mu m$. The maximum of the emission spectrum of the dye is at a wavelength $\lambda = 645$ nm. Two different objectives have been used, x25/0.6 and x40/1.3. The slide preparation has been done in such a way that the beads were closely against the coverslip to avoid a mismatch in refraction index.
Figure 2.7: A one dimensional plot of a fluorescent bead acquired at different positions, z = 0.0 µm, 0.2 µm, 0.4 µm, 0.8 µm, 1.6 µm. Two objectives have been used, x40/1.3 and x25/0.6. The diameter of the bead is 0.2 µm.

Figure 2.7 shows that defocusing reduces the maximum intensity significantly and at the same time increases the size of the bead. The effect of defocusing is larger for high NA objectives. If the bead is 1.6 µm out-of-focus and a x40/1.3 objective is used, the maximum intensity is reduced by a factor of 2. The size of the bead is 0.20 µm² which is significantly larger than 0.13 µm² of the in-focus dot. The size of a dot is defined as the number of pixels, expressed in µm², at half the maximum intensity of the dot. Figure 2.8 shows the theoretical PSF as a function of the focus position. The maximum intensity of
the PSF decreases much faster than the measured maximum intensity of the bead. At $z = 0.6 \, \mu m$ the maximum intensity of the PSF is only 12% of the in-focus PSF. Although the beads are relatively small (one third of the wavelength) they can not be approximated by a point source. The influence of the focus position when a bead is imaged is significantly less than when a point source is imaged. The effect of defocusing is not only related to the objective that is used, but also related to the application. In the case of FISH dot counting, the depth-of-focus could be defined as the distance $\Delta z$ over which the normalized maximum intensity of a dot is above a certain level. For example, the maximum intensity of a dot must always be above 80% of the maximum intensity of the in-focus dot. A more detailed discussion and experimental results about the relation between features and defocusing are presented in chapter 4.

![Normalized intensity graph](image)

**Figure 2.8:** The theoretical point spread function for different degrees of defocusing. The maximum intensity of the in-focus PSF is normalized to one. The maximum intensity of the PSF reduces significantly as a function of the focus position.

The image brightness, resolution and depth-of-focus are directly related to the numerical aperture and not to the lens magnification. The lens magnification becomes important when the sampling density is considered (see section 2.4.3). Considering the brightness and resolution one should choose an objective with a high numerical aperture. But this will result in a small depth-of-focus. Metaphase spreads are in generally very thin. The thickness of interphase cell nuclei is more related to the specimen and preparation technique (Rijke et al 1996). Depending on the flatness of the specimen, a required (minimum) depth-of-focus determines an upper bound for the numerical aperture.
2.3 Imaging Device

The fluorescence microscope images have to be digitized and stored in computer memory before further automated analysis is possible. In fluorescence microscopy we have to deal with low light intensities. Imaging a Spectrum Orange CEP 8 probe with a Photometrics KAF 1400 camera and a x40/1.3 objective, we have to integrate for one second to accumulate 16,000 electrons in one CCD well (half the dynamic range). Comparing with room-level illumination, the Sony XC-77-RR-CE video camera accumulates about 20,000 electrons (again half the dynamic range) in 40 msec. (video rate). The weak intensity of a fluorescent specimen requires that the camera can integrate for a few hundred msec. to more than several seconds. The integration time depends on the NA, the brightness of the specimen, the camera sensitivity, and the required signal-to-noise ratio. The goal of this section is to get a better insight into the performance of the cameras and to assist one in selecting a camera for a specific application in fluorescent microscopy. A model is given that describes the photometric response of a camera. Two cameras are characterized and the results are compared. The evaluation methodology, itself, has been presented elsewhere (Mullikin et al 1994) and only the main results will be described and applied here. Signal-to-noise ratio (SNR), sensitivity, efficiency, linearity, and dark current are discussed.

2.3.1 CCD cameras

In most cases a CCD camera is used as imaging device. A variety of CCD cameras are available. These cameras differ in performance, functionality, and price. Low cost cameras are often video based cameras. It is possible to modify a normal video camera in such a way that it can integrate on the CCD chip (Vrolijk et al 1993). More expensive cameras are generally slow-scan cooled CCD cameras. Such a camera has the required characteristics for fluorescence microscopy. These cameras are frequently used in astronomy where an integration time of several minutes is normal (Leach 1987).

A CCD element consists of a two-dimensional matrix of photosensitive sites, the electron wells. This matrix is called the parallel register. In the operation of a CCD camera we distinguish two stages: the integration time in which the exposure takes place; and the readout time. During integration, photons produce electrons in the CCD wells. These electrons are referred to as photoelectrons. When sufficient photoelectrons are accumulated, the image has to be transferred to computer memory. The readout procedure depends on the type of CCD chip (see figure 2.9). Slow scan CCD cameras are in general based on a full frame CCD. Charge stored in the parallel register is shifted, in parallel, towards a serial register. Each row that is transferred to the serial register, is then shifted, pixel by pixel, to the pre-amplifier and analog-to-digital converter (ADC).
Image acquisition

- Full frame CCD
- Interline transfer CCD

Figure 2.9: Two CCD architectures: a full frame CCD and an interline transfer CCD. The readout procedure depends on the type of the CCD chip.

After the serial register is emptied the whole cycle of a parallel shift followed by a serial readout is repeated until the parallel register is emptied. The readout time depends on the readout rate and the dimension of the CCD element. A typical readout time of a slow scan CCD camera can vary between a few hundred milliseconds to a few seconds. To avoid smearing during the readout phase, a shutter must be included to block the light. Another CCD type, that is often used in video cameras, is the interline transfer CCD. This CCD consists of alternating columns of light sensitive pixels and light insensitive pixels. During readout, the charge of the light sensitive pixels are transferred, in parallel, to the light insensitive pixels. After this first step the image is transferred to the ADC in the same way as with a full frame CCD. Because the image is transferred immediately after the integration to the insensitive area no shutter is required. The disadvantage of such a CCD element is that only a part of the area is light sensitive. A longer integration time is required to accumulate enough photoelectrons.

Intensified CCD cameras are also used to deal with low light intensities (Reynolds 1980, Wick 1987). Such a system does not have the proper characteristics, because the intensifier causes a boost of the photon shot noise and reduces the spatial resolution. When integration is not possible, for example when monitoring a fast dynamic process, intensified cameras can be a good solution. Intensified cameras are not discussed in this thesis.

FISH applications make use of different dyes to label different probes simultaneously. The different dyes are distinguished based on their color. A color camera can be used to obtain the color information. A single chip color camera is not suitable for quantitative microscopy. Due to a mosaic pattern or stripe filters on the CCD to select the RGB colors
(red, green, and blue), only one third of the pixels is used for one channel. The pixel spacing becomes large and only one third of the light is collected. A three chip RGB color camera does not have this disadvantage. A chromatic prism block in front of the three CCD chips splits the light into three channels. The spectral characteristics of the three channels are shown in figure 2.10. The three images are acquired simultaneously. The disadvantage of such a camera is that the integration time is equal for all channels. If the brightness of dyes differs too much, saturation can cause problems.

![Relative Transmission Graph](image)

**Figure 2.10:** The spectral characteristics of the three channels, red, green, and blue of a RGB color camera (data from Andover Corporation, Salem, NH).

So far we have only discussed the cameras but not how the signal is actually digitized and stored in computer memory. With a slow-scan camera, the interface to the computer is normally included. The output of a video based camera is a standard video signal. A frame grabber must be used to digitize the video signal. In this thesis the combination of a video CCD camera and a frame grabber is called a camera system or imaging device.

This section presents the characterization of two cameras. Table 2.2 gives the manufacturers specifications for the two relevant cameras: the Photometrics KAF-1400, and the Sony XC-77-RR-CE. They are both monochrome cameras. The Sony XC-77-RR-CE is a video based camera. We have used a *QuickCapture* frame grabber (Data Translation Inc., Marlboro, USA) to digitize the images. The Photometrics camera is a slow-scan cooled CCD camera. Beside the different types of the CCD chips, there are also differences in functionality. The Photometrics camera is provided with a Peltier cooling element to suppress the dark current. The Sony camera is in principle a normal video camera, and therefore gamma correction and auto gain are included. These features
are not desirable for quantitative microscope and should be turned off. The Photometrics camera has the possibility of pixel binning. Binning is the process of combining adjacent pixels on the CCD into one larger pixel and this can be used to increase the signal but reduce the spatial sampling frequency. If, for example, a 4 x 8 binning is used, a series of "superpixels", consisting of 32 original pixels each, is used. The area covered by a typical superpixel would then be 27.2 x 54.4 μm². If binning is used the readout time is shorter. In this example the readout time would be 0.8 sec. instead of 2.7 sec without binning.

Table 2.2: The manufacturer's specifications for the Photometrics KAF 1400 camera and the Sony XC-77-RR-CE camera.

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<thead>
<tr>
<th>Camera</th>
<th>Photometrics</th>
<th>Sony</th>
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<tr>
<td>Manufacturer Type/ model</td>
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<td>XC-77-RR-CE</td>
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</tr>
<tr>
<td><strong>Performance</strong></td>
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<td></td>
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<tr>
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</tr>
<tr>
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</tr>
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</tr>
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<td></td>
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<tr>
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<td>Data Translation, 8 bits</td>
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<td>Macintosh, Nubus</td>
</tr>
<tr>
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<td>14 MHz</td>
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<tr>
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<tr>
<td>UV coating</td>
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</tr>
<tr>
<td>Binning</td>
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<td>No</td>
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</tbody>
</table>
### 2.3.2 Photometrics response

Considering one CCD element at position \((x,y)\), a pixel measures the number of incident photons expressed in analog-to-digital units (ADU). For a given photon flux \(\phi_p\) and an integration time \(t\), the measured pixel value \(I(x,y)\) is given by:

\[
I(x,y) = g_e \int_{\lambda} t \cdot A \cdot \tau_A \cdot QE(\lambda) \cdot \tau_e(\lambda) \cdot \Phi_e(\lambda) \cdot d\lambda = g_e \cdot N_e
\]  

(2.7)

The term \(A \cdot \tau_A\) is the light sensitive area of a pixel where \(A\) is the area and \(\tau_A\) is called the fill factor of the CCD element. For example, with an interline transfer CCD, only half the pixel area is light sensitive and the fill factor will be about 0.5. The subscript \(c\) denotes the channel of the camera where \(\tau_e\) is the spectral transmission response of the filters that are part of that channel. This could be an infra-red (IR) filter or the spectral characteristics of the three channels of a color camera (see figure 2.10).

**Figure 2.11:** Typical quantum efficiency curve of a frontside illuminated CCD. The dashed line shows the improved quantum efficiency when a special coating is applied. This data has been published by (Aikens 1992).

\(QE(\lambda)\) is the quantum efficiency of the CCD chip, which is the probability that an incident photon is converted to an electron. A typical example of the quantum efficiency as function of the wavelength \(\lambda\) is shown in figure 2.11 (Aikens 1992). Near the IR the quantum efficiency approaches 40% whereas in the ultra violet (UV) it is almost zero. The maxima of the emission spectrum of the two dyes that we have used — DAPI and Spectrum Orange — are at 461 nm and 615 nm and result in a quantum efficiency of 11%
and 26%, respectively. To increase the UV response it is possible to coat the CCD surface with a thin layer that absorbs UV radiation and emits at a longer wavelength. The material of the coating is called lumogen (Kristianpoller and Dutton 1964). The commercial name used by Photometrics is Metachrome II. The dashed line in figure 2.11 gives the quantum efficiency when such a coating is applied.

The integral of eq. 2.7 defines the number of electrons $N_e$ that are accumulated in the CCD well. The electronic gain $g_e$ of the camera is defined as the number of ADU's per electron. For simplicity the subscript $c$, and the position $(x,y)$ are not included in further equations, $I$ will be the intensity of one pixel at position $x,y$ of channel $c$.

2.3.3 Camera noise

Equation 2.7 describes the response of an ideal camera. In practice the measured intensity will be disturbed by different noise sources. Photon shot noise, thermal noise, readout noise, and quantization noise will be discussed.

- Photon shot noise — This fundamental type of noise is caused by the quantum nature of light. The noise problem arises from the statistical nature of photon production. Accumulating electrons in a CCD well over an interval $t$ can be described as a counting process and obeys Poisson statistics. If an average of $N_e$ electrons are collected in an observation window of duration $t$, the variation of the measured intensity is $\sigma^2 = N_e$. Converting this to ADU$^2$, the variation of the measured intensity of one pixel due to the photon shot noise is given by:

$$\sigma_p^2 = I \cdot g \quad \text{(ADU}^2\text{)}$$  \hspace{1cm} (2.8)

This noise source can never be eliminated and thus forms the limiting case when all other noise sources are reduced to a negligible level.

- Thermal noise — Electrons are not only produced by photons, but also by thermal energy. This is called dark current. Electrons can be freed from the material itself through thermal vibration. These electrons can not be distinguished from photon induced electrons. Dark current causes an offset of the measured intensity and will reduce the dynamic range of the camera. The dark current $\Phi_d$ is defined as the number of thermally induced electrons per time interval per pixel. Besides the offset caused by the dark current there is also a stochastic component. The probability distribution of the number of thermal electrons is also a Poisson process. The variation per pixel, again in ADU$^2$, due to the dark current is given by:

$$\sigma_d^2 = t \cdot \Phi_d \cdot g^2 \quad \text{(ADU}^2\text{)}$$  \hspace{1cm} (2.9)
The dark current $\Phi_d$ is an increasing function of the temperature. By cooling the CCD chip it is possible to reduce the dark current significantly. There are alternative techniques for suppressing the dark current. The dark current generation can be effectively suppressed by an electronic method that is called Multi-Pin Phasing (MPP). MPP can reduce the dark current by 1 or 2 orders of magnitude (Burkey and Chang, Princeton-Instruments 1995). The disadvantage of this technique is reduced charge storage capacity of the pixels, typically 1.5 times smaller. Video based cameras are often provided with auto-dark current subtraction. The average dark current is estimated from a row of CCD elements that are not exposed. This value is subtracted from each pixel before the ADC. Another possibility to correct for the dark current is flat field correction. This technique is applied after the digitization of the image and will be discussed in Section 2.3.5. Although auto-dark current subtraction and flat field correction do reduce the dark current offset, they do not reduce the dark current variation, and the dynamic range will be reduced.

- Readout noise — The readout noise originates in the output preamplifier of the CCD. The preamp amplifies the charge content of one pixel that is transferred to it by the shift register, after which the signal is digitized by an ADC. The readout noise depends on the quality of the analog electronics. The noise is independent of the signal and a function of the readout rate (Janesick et al 1987). Manufacturers commonly express the readout noise, if specified, in electrons root-mean-square (RMS, or standard deviation $\sigma_r$). Due to the slow readout rate, the specified readout noise of the Photometrics camera is only 11.7 electrons. The readout noise is a lower bound of the camera noise and determines the lowest detectable signal.

- Quantization noise — Quantization noise is caused by the amplitude quantization process. The ADC digitizes the analog amplitude of a pixel into a discrete number between 0 and $2^b-1$, where $b$ is the number of bits of the ADC. The noise is additive and independent of the signal, if the number of levels is larger than 16 ($b > 4$). The variance associated with the noise becomes:

$$\sigma_q^2 = \frac{1}{12} \text{ (ADU}^2)$$

(2.10)

This is the variance of a uniform distribution between -0.5 and 0.5. The SNR as a result of the quantization process is given by:

$$SNR_q = 6 \cdot b + 11 \text{ (dB)}$$

(2.11)

For $b \geq 5$ bits and using the full dynamic range, this means a $SNR_q \geq 41 \text{ dB}$. In other words, if only 32 discrete levels are used to digitize the signal, the SNR is still above 41 dB. In general, all camera systems are using an ADC with $b \geq 8$ bits. Video frame grabbers are generally using 8 bits. Using a slow scan CCD camera the number of bits
can increase to more than 16 bits (Princeton-Instruments 1995). Quantization noise can usually be ignored, as the noise is negligible compared to other sources.

- Signal-to-noise (SNR) — The four different noise sources will contribute to the total camera noise. Assuming that each noise source is independent, the variance of the camera noise is given by:

\[ \sigma_{\text{camera}}^2 = \sigma_p^2 + \sigma_d^2 + \sigma_r^2 + \sigma_q^2 \quad \text{(ADU}^2\text{)} \]  

(2.12)

The SNR of a pixel is then defined as:

\[ \text{SNR} = 20 \cdot \log \left( \frac{I(x, y)}{\sigma_{\text{camera}}} \right) \quad \text{(dB)} \]  

(2.13)

The ideal camera would have a negligible dark current, readout noise and quantization noise. Because the photon shot noise can not be eliminated, the upper bound of the SNR, using eqs. 2.8 and 2.13, is given by:

\[ \text{SNR}_{\text{ideal}} = 10 \cdot \log \left( \frac{I(x, y)}{g} \right) \quad \text{(dB)} \]  

(2.14)

where \( I(x, y)/g \) is equal to the number of photon induced electrons in the CCD well. The maximum SNR of an ideal camera is determined by the full well capacity of the CCD element. For example, the full well capacity of the Photometrics camera is about 32,000 electrons and in the ideal case that will result in a maximum SNR of 45 dB.

2.3.4 Camera pixel variation

So far we have discussed the photometric response of a single pixel. In practice the variation between pixels of a uniform illuminated image is larger then the variation caused by the different noise sources. The pixel variation is caused by a different response of the individual CCD wells to the same amount of light. The sensitivity can vary among the pixels. The variation between the pixels is not a stochastic process like the photon shot noise or readout noise but is deterministic. Therefore it is possible to reduce the variation between the pixels after image acquisition using flat field correction. If flat field correction is not applied to an image, the pixel variation can be considered as an extra noise source.

Besides small variations between the pixels some pixels can be damaged. CCD chips usually contain various defects. These errors cannot be recovered digitally. Two important defects are dark points and hot spots. Dark points represent pixels with almost negligible response in comparison to other pixels. Hot spots are pixels with a much higher dark current compared to other pixels. These pixels will be saturated after a short integration time. Some manufactures of scientific cameras offer the possibility to select a
CCD chip with a specified quality. This is in general not the case with video based cameras. The grade of a CCD chip is commonly defined as a measure of the maximum number of imperfect pixels.

2.3.5 Flat field correction

Flat field correction is applied after image acquisition. It is not a part of the imaging device. Flat field correction is included in this section because it is directly related to the camera characterization and it gives the possibility to improve the image quality significantly. This technique reduces or removes the effects of dark current, pixel variation, and also non-uniform illumination. The model of an acquired image is given by:

\[ I(x, y, t) = D(x, y, t) + C(x, y) \cdot F(x, y) \cdot t \]  

(2.15)

where \(D(x, y, t)\) is the offset and \(C(x, y)\) is a conversion factor. The offset is the sum of the dark current and the bias of the camera. \(F(x, y)\) is the number of fluorescent molecules at position \((x, y)\). The conversion factor describes the relation between the number of fluorescent molecules and the measured intensity. The brightness of a specimen at position \((x, y)\) is proportional to the amount of the fluorescent dye and the excitation intensity. In the ideal case the measured intensity is proportional to the number of fluorescent molecules, and has a zero offset. But in practice the offset is not zero and both the offset and conversion factor are a function of the position. The conversion factor depends, among other things, on the electronic gain \(g\), quantum efficiency \(QE\), transmission coefficient \(\tau\), excitation spectrum, the fluorescent dye and the optical system. Although it is not possible to measure or calculate the exact value of the overall conversion factor, it is possible to make \(C(x, y)\) independent of the position. The corrected image should be unbiased, proportional to \(F(x, y)\), and the conversion independent of the position.

Flat field correction makes use of two calibration images – \(Dark(x, y)\) and \(White(x, y)\). The \(Dark\) image is acquired by closing the excitation shutter which result in \(C(x, y) \cdot F(x, y) = 0\), and the \(Black\) image becomes \(Black(x, y) = D(x, y)\). The \(White\) image is acquired by imaging a uniform FOV, in other words \(F(x, y) = constant\). If the \(Dark\) image is subtracted from the \(White\) image the resulting image becomes proportional to the conversion factor \((White(x, y, t) - Dark(x, y, t) = C(x, y) \cdot constant)\). Flat field correction then becomes:

\[ I_{ff}(x, y, t) = constant \cdot \frac{I(x, y, t) - Dark(x, y, t)}{White(x, y, t) - Dark(x, y, t)} \]  

(2.16)

If the \(constant\) term is equal to the average intensity of the \(White\) image minus the \(Black\) image, the average intensity of the flat field image becomes equal to average intensity of the original image.
Image acquisition

The Dark image and White image have to be acquired each time a new experiment starts. Changing the imaging setup can influence the result of the White image. To suppress the noise of the two calibration images we take the average over 10 images. The SNR of the calibration images will then be significantly higher than the original image. Averaging 10 images results in a 10 dB higher SNR of the calibration images. In that case the SNR of the corrected image is comparable to the SNR of the original image and is not reduced by the calculations. To image a uniform field one can use a slide that contains a homogeneous solution of the fluorescent dye. The solution should be a thin layer. If the layer is too thick, out-of-focus light will influence the result. Therefore uranyl glass or plastics, which are usually a few millimeter thick, should not be used for this purpose.

2.3.6 Camera evaluation
The performance of the Photometrics KAF 1400 and the Sony XC-77-RR-CE has been evaluated. The methods to characterize a CCD camera are briefly discussed. A more detailed discussion has been reported in (Mullikin et al 1994). The following properties have been measured: linearity, signal-to-noise ratio, dark current, sensitivity, and relative efficiency. The two cameras that have been evaluated are more or less the extremes of the whole range of CCD cameras. The Sony camera is a low-cost, video-based B/W camera, that is modified so that it can integrate on the CCD chip. The Photometrics camera is a very expensive slow-scan cooled CCD camera with an excellent performance.

The microscope has been setup for bright-field illumination. A green interference filter (500FS10-50, Andover Corporation, Salem, NH, USA) has been used to select the illumination wavelength, with the exception of measuring the relative efficiency as a function of the wavelength. A x40/1.3 objective has been used to image a uniform field. We have used a test slide (Press-Pro21, the Press project) that contains, among other things, homogeneous regions of different neutral densities. Other objects on the test slide have been used for focusing. A sequence of images have been acquired of a uniform FOV for both cameras. The integration time and illumination brightness have been varied. For each combination of the integration time and illumination brightness, two images have been acquired for both cameras. The integration time has been varied from 0.1 s to 8.0 s. This set of images has been used to characterize the photometric response of the cameras.

2.3.7 Linearity
It is obvious from eq. 2.7, that the relationship between the number of incident photons and the measured output signal should be linear. Linearity is indicated by the coefficient of regression, $R^2$, calculated from integration time versus the average intensity of the images for a constant photon flux. These values are taken directly from the set of images acquired with the same illumination brightness and different exposure times. Below
saturation, the CCD cameras are linear, resulting in $R^2 > 0.9999$ over the whole dynamic range.

2.3.8 Signal-to-noise ratio
To calculate the signal-to-noise ratio the following equation is used:

$$SNR = 20 \cdot \log \left( \frac{\bar{I}}{s} \right) \text{ (dB)}$$  \hspace{1cm} (2.17)

where $\bar{I}$ is the mean of the image and $s$ is the estimated standard deviation of the image. Depending on the definition of the standard deviation, two different signal-to-noise ratios can be distinguished – pixel SNR and image SNR. The pixel SNR is related to the variation of the pixels between two or more independent images. The variance is then defined as:

$$s_p^2 = \frac{1}{2} \text{var}(I_1 - I_2)$$ \hspace{1cm} (2.18)

where $I_1$ and $I_2$ are independent, uniformly illuminated images, with the same average intensity $\bar{I}$. The image SNR, which is related to variation between the pixels within one image, is calculated using the following definition of the variance:

$$s_i^2 = \text{var}(I)$$ \hspace{1cm} (2.19)

The mean and variance are calculated over a region of 100 by 100 pixels in the center of the image.

**Figure 2.12:** The pixel SNR and the image SNR for the Photometrics camera and the Sony camera. The gray lines are the ideal SNR's of both cameras, assuming that they are photon limited.
Figure 2.12 shows the pixel SNR and the image SNR for both cameras. The ideal SNR is defined by eq. 2.14, assuming that the camera is photon limited. We have used the relative sensitivity, which is the inverse of the electronic gain, given in table 2.3 to obtain the ideal SNR. The measured maximum pixel SNR is about 45 dB for both cameras. The image SNR curve shows clearly that the variation between the pixels is larger than the variation caused by the different noise sources. If an image is not flat field corrected, the maximum image SNR is 10 dB below the pixel SNR. Figure 2.13 illustrates the effect of flat field correction. The image SNR has been measured again after flat field correction. The image SNR of the flat field image approximates the pixel SNR of the original image. The difference is about 2 dB at the maximum intensity. Although the SNR's of the calibration images are about 10 dB higher than the original image, the SNR of the flat field image is slightly reduced.

![SNR Graph]

**Figure 2.13:** The Image SNR of the Photometrics camera after flat field correction. The Image SNR then approximates the Pixel SNR of the original image.

Considering the SNR curves of the two cameras, they are more or less comparable. Only the curve of the Sony camera seems to be more noisy. Small variations in the electronic gain during the experiments can be an explanation for the fluctuation of the SNR curve. Calculating the pixel SNR, we assumed that the average intensities of the two images are equal. The standard deviation of the estimated average intensity is given by $\sigma_I = \sigma_{\text{camera}} / \sqrt{n}$, where $n$ is the number of pixels that are used to estimate the mean. With 18% of the 1020 image pairs, that have been acquired with the Sony camera, the
absolute difference between the average intensity of the two images exceeded a $3 \cdot \sigma_i$ interval. This is much higher than we would expect, if the electronic gain were stable. We did not observe this effect with the Photometrics camera.

2.3.9 Dark current

Dark current is defined as the rate of induced electrons per pixel from all sources other than photons. Measuring the dark current is relatively simple and does not require an optical setup. A number of images have been acquired with different integration times and without any illumination. The dark current can be estimated using the average intensity or the pixel variance (eq. 2.18) of the dark images as function of the integration time. Figure 2.14 shows the average intensity and the variance (eq. 2.18) of the dark images as function of the integration time. These results have been obtained with the Photometrics camera. Both the mean and variance seem to be independent of the integration time. The dark current of the Photometrics camera is negligible within the range of the measurements. This is in agreement with the specified dark current of 0.03 e$^-$/sec. An integration time of 8 sec. would result in an offset of the measured intensity of 0.03 ADU (electronic gain of 8.0 e$/ADU$, see table 2.2).

![Figure 2.14: The average intensity and the pixel variance (eq. 2.18) of the dark images as function of the integration time. The results have been obtained with the Photometrics camera. Both the mean and variance are independent of the integration time within the range of the measurements.](image)

The Sony camera is provided with auto dark current subtraction. Therefore the average intensity of the dark images cannot be used to estimate the dark current. Figure 2.15 shows the pixel variance (eq. 2.18) and image variance (eq. 2.19) of the dark images. The pixel variance is stable for an integration time below the 8 sec. The average dark current
over the entire image is not significant. The image variance is not independent of the integration time. This effect is caused by the hot spots. A limited number of pixels have a significantly higher intensity. Because the number of hot spots is small with respect to the total number of pixels, the influence on the pixel variance is small. The amount of dark current from the hot spots is not significant when the average dark current per pixel is estimated. The high intensity of the hot spots however, will have a contribution to the image variance.

![Graph](image)

**Figure 2.15:** The pixel variance and image variance of the dark images obtained with the Sony camera. The pixel variance is stable for an integration time below 8 sec. The image variance is not independent of the integration time due to the hot spots.

### 2.3.10 Sensitivity

There are two different ways to define the sensitivity of a camera. First, the absolute sensitivity that determines the minimum number of detectable photoelectrons. Secondly, we can describe the number of photoelectrons necessary to change one ADU. This is called the relative sensitivity.

- **Absolute sensitivity —** The detectability of the minimum number of photoelectrons is determined by the noise characteristics of the camera. To be able to detect a certain amount of photoelectrons, the response of the camera must be above the noise level. To ensure the detectability of a signal, one could say that the $3\times \sigma$ level would be the minimum detectable signal. Where $\sigma$ is the standard deviation of all noise sources with
the exception of photon shot noise. The absolute sensitivity is defined as the standard deviation of the camera. Because the dark current and quantization noise are negligible, the absolute sensitivity is determined by the readout noise. The readout noise is estimated by calculating the pixel variance of a dark image. Because there is no illumination the photon shot noise will be zero and the resulting variance is caused by the readout noise.

![Graph showing pixel variance as a function of average intensity for Photometrics and Sony cameras.](image)

**Figure 2.16:** The pixel variance as a function of the average intensity. The variance should be linear with the intensity. Then the slope of the resulting straight line determines the electronic gain.

- Relative sensitivity — The number of photoelectrons to change one ADU is equal to the inverse of the electronic gain. The measurement of the sensitivity is based on the variance of the camera as a function of the intensity. Eq. 2.12 in combination with eqs. 2.8, 2.9, and 2.10 shows that for a given integration time the variance should be linear with the intensity and the slope of the resulting straight line determines the electronic gain. Figure 2.16 gives the pixel variance as function of the average intensity for both cameras. The pixel variance of the Photometrics camera is linear with average intensity. A linear fit result in a coefficient of regression $R^2 > 0.9999$ and the sensitivity is equal to the slope of the linear fit. The variance of the Sony camera is not linear with the average intensity. The coefficient of regression of a linear fit becomes $R^2 = 0.744$. A second order polynomial fit results in much higher coefficient of regression $R^2 = 0.994$. The non-linear relationship between the average intensity and the variance is possibly caused by a non pixel synchronous sampling of the frame grabber. The output of the CCD element is first converted to an analog video signal and then the video signal is re-sampled by the frame grabber. Because the number of pixels per line is not equal to the number of CCD pixels per row, the positions of the pixels of the frame grabber do not correspond to the position
of the CCD pixels. Jitter of the video signal will cause small variation in the spatial sampling position of the resulting image with respect to the position in the parallel shift direction of the CCD element. The conversion between the discrete pixels and analog video signal also involves a low-pass filtering of the image in the parallel shift direction. Therefore the measured intensity of a single pixel will not be independent from his neighborhood pixels.

Table 2.3: The absolute and relative sensitivity for both cameras. The absolute sensitivity is defined as the standard deviation of the camera noise with the exception of the photon shot noise. The standard deviation is expressed in e⁻ and ADU.

<table>
<thead>
<tr>
<th>Sensitivity</th>
<th>Photometrics</th>
<th>Sony</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absolute (e⁻)</td>
<td>11.3 ± 0.2</td>
<td>262. ± 13.</td>
</tr>
<tr>
<td>Absolute (ADU)</td>
<td>1.41 ± 0.03</td>
<td>0.61 ± 0.05</td>
</tr>
<tr>
<td>Relative (e⁻/ADU)</td>
<td>8.007 ± 0.019</td>
<td>109.2. ± 5.2</td>
</tr>
</tbody>
</table>

Because the Sony camera is not photon limited we have used a different approach to estimate the relative sensitivity. The Photometrics camera has been used to calibrate the photon flux. First an image is acquired with the Photometrics camera. Then the Photometrics camera is replaced by the Sony camera, without changing the microscope setup, and a second image is acquired. The microscope has been setup for bright field illumination. An interference filter has been used to select the illumination wavelength $\lambda = 500$ nm. Eq. 2.7 can now be re-written as:

$$QE(\lambda = 500nm) \cdot \tau_c(\lambda = 500nm) \cdot \phi_p(\lambda = 500nm) = \frac{I(x,y)}{g_c \cdot \tau_A \cdot A \cdot t} \quad (2.20)$$

Assuming that the quantum efficiency $QE$ and the spectral transmission response $\tau_c$ are equal for both cameras, the left side of eq. 2.20 will be constant for a given photon flux $\Phi_p$. Given the gain of the Photometrics camera, it is now possible to estimated the gain of the Sony camera with the following formula:

$$g_{sony} = \frac{\bar{I}_{sony} \cdot \phi_{phot} \cdot A_{phot} \cdot \tau_{A,phot} \cdot t_{phot}}{\bar{I}_{phot} \cdot A_{sony} \cdot \tau_{A,sony} \cdot t_{sony}} \quad (2.21)$$

The relative sensitivity is the inverse of the electronic gain $g_{sony}$. With $g_{phot} = 0.1249$ ADU/e⁻ (table 2.3), $A_{phot} = 6.8x6.8$ $\mu$m², $\tau_{A,phot} = 1$, $A_{sony} = 11x11$ $\mu$m², and $\tau_{A,sony} = 0.5$ (interline transfer CCD) eq. 2.21 becomes:

$$g_{sony} = 9.545 \cdot 10^{-2} \cdot \frac{\bar{I}_{sony} \cdot t_{phot}}{\bar{I}_{phot} \cdot t_{sony}} \quad (2.22)$$

where $\bar{I}_{sony}$ and $\bar{I}_{phot}$ are the average intensities of two uniformly illuminated images acquired with an integration time $t_{sony}$ and $t_{phot}$.
Table 2.3 gives the absolute and relative sensitivity of the Photometrics camera and Sony camera. The assumption that the quantum efficiency and spectral transmission response are equal for both cameras is not completely correct and will cause a small bias for the estimated relative sensitivity of the Sony camera. For example, the Photometrics camera is mounted on the microscope with an adapter that contains an extra relay lens. The spectral transmission response will therefore be slightly lower.

2.3.11 Relative efficiency
The results so far showed that for each camera it is possible to accumulate enough photons until it achieves a certain SNR. One important question remains, how long do we have to integrate for a given photon flux? Differences of the quantum efficiency or fill factor of the CCD well will lead to different integration times to achieve a certain SNR. The efficiency has been defined as the integration time that is necessary to achieve a certain SNR for a given photon flux. Because we do not have a calibrated illumination source that gives a specified photon flux, we can only measure the efficiency relative to another camera.

![SNR vs Integration Time](image)

**Figure 2.17:** The SNR as function of the integration time for a constant photon flux. The integration time of the Sony camera is about twice the integration time of the Photometrics for a SNR of 40 dB.

We have measured the SNR as function of the integration time for a constant photon flux. The microscope setup has not been changed during the measurements for both cameras. First the SNR of the Photometrics camera has been measured. The Photometrics camera has been replaced by the Sony camera and again the SNR has been measured as a function...
of the integration time. The measurements have been done at different illumination wavelengths, $\lambda = 400, 500, 600, \text{ and } 700 \text{ nm}$. Because the pixel size of the two cameras are different the integration time is normalized with respect to the pixel area. Figure 2.17 shows the result measured at a wavelength $\lambda = 500 \text{ nm}$. It is obvious that we have to integrate much longer with the Sony camera than with the Photometrics camera, to reach a certain SNR. Table 2.4 gives the relative efficiency of the Sony camera for different illumination wavelengths.

Table 2.4: The relative efficiency of the Sony camera for different illumination wavelengths. The efficiency is relative to the efficiency of the Photometrics camera. The relative efficiency is defined as the ratio between the integration time of the Photometrics camera and the integration time of the Sony camera, to achieve a SNR of 40 dB. The integration time is corrected for the different pixel size of the two cameras.

<table>
<thead>
<tr>
<th>Wavelength</th>
<th>Relative efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>400 nm</td>
<td>0.39$\times 10^{-2}$</td>
</tr>
<tr>
<td>500 nm</td>
<td>0.43</td>
</tr>
<tr>
<td>600 nm</td>
<td>0.24</td>
</tr>
<tr>
<td>700 nm</td>
<td>0.65$\times 10^{-2}$</td>
</tr>
</tbody>
</table>

The efficiency is relative to the efficiency of the Photometrics camera and is defined as the ratio between the integration time of the Photometrics camera and the Sony camera, to achieve a SNR of 40 dB. The low relative efficiency of the Sony camera at $\lambda = 400 \text{ nm}$ is caused by the absence of a special coating to improve the UV response. The efficiency at $\lambda = 500 \text{ nm}$ is still a factor two less than with the Photometrics camera. The interline transfer CCD chip has a fill factor of about 50% due to the light in sensitive columns. The relative efficiency decreases at a higher wavelength to almost zero at $\lambda = 700 \text{ nm}$. This is possibly caused by the IR filter of the Sony camera.

2.3.12 Conclusions
The Photometrics camera has a SNR that is photon limited over the entire range. The Sony camera does not show a linear relation between the average intensity and pixel variance and therefore the camera is not photon limited. Although the Sony camera is not photon limited, the maximum SNR is only slightly below the maximum SNR of the Photometrics camera. The maximum SNR of the Photometrics camera is limited by the full well capacity of 32,000 electrons and results in a maximum SNR of 45 dB. The full well capacity of the Sony camera is 40,000 electrons. This could result in a theoretical maximum SNR of 46 dB, if the camera would have been photon limited.

The average dark current is not significant for both cameras for an integration time below the 8 sec. The average intensity and pixel variance of the dark images are independent of the integration time over the entire range of the measurements. This result would lead to
the conclusion that cooling is not necessary because the Sony camera is a non-cooled camera. But cooling also reduces the effect of the hot spots. The Sony camera contains a limited number of pixels with a much higher dark current compared to other pixels. Because the number of hot spots is limited, they do not make a significant contribution to the average dark current. Of course if the intensity of a small dot has to be measured a single hot spot can disturb the result. The Photometrics camera does not have these hot spots.

The relative sensitivity is dominated by the electronic gain. Because the Photometrics camera has a lower dynamic range (32,000 e⁻ versus 40,000 e⁻) and uses an ADC with more bits (12 bits versus 8 bits), the sensitivity is much higher than the sensitivity of the Sony camera.

The most important difference between the two cameras is the efficiency. The interline transfer CCD chip of the Sony camera is not preferable when speed is an important issue. The fill factor is only 50% and results in a two times longer integration time. The special coating of the Photometrics is necessary when one is using a dye with an emission spectrum near the UV. The efficiency of the Photometrics camera is 250 times higher than the Sony camera at $\lambda = 400$ nm. The IR filter of the Sony camera reduces the efficiency at $\lambda = 600$ nm and 700 nm. We have included an IR filter in the excitation path which blocks the IR radiation sufficient. Our experience with the Photometrics camera is that the IR radiation that reaches the sensor is low and therefore an extra IR filter in the emission path is not necessary.

It is difficult to draw definitive conclusion from the results presented in this section. The Photometrics camera seems to be more suitable for quantitative microscopy because the SNR is photon limited, has a high sensitivity and has a much better efficiency. Other advantages of the Photometrics camera are the large dimension of the chip in combination with a small pixel size and the possibility of binning. There is one drawback, the long readout time of 2.7 sec. caused by the slow readout rate of 2 μs per pixel. There are other cameras that have a higher readout rate and comparable performance. For example, the Xillix Micro Imager. This camera uses the same CCD chip as the Photometrics camera but has a 16x faster readout rate (variable readout rate 0.5, 2, 4, 8 MHz). Although it has a faster readout rate and the camera is not cooled, the pixel SNR of the Xillix camera is comparable to the SNR of the Photometrics camera (Mullikin et al 1994). The absence of cooling will cause hot spot like the Sony camera. Other newer Photometrics cameras also have an increased readout rate.

Finally one should notice that the performance of the Sony camera is not only determined by the performance of the camera itself, but also by the frame grabber. In other words we
did not measure the performance of the Sony camera but we have measured the performance of the Sony camera in combination with the QuickCapture frame grabber.

2.4 Automated scanning

The whole imaging system — fluorescence microscope and camera — has to be configured in such a way that it is capable of scanning slides automatically. Besides the image quality, the scanning speed is an important feature of an automated scanning system. It will not always be possible to meet all requirements for a specific application. Some requirements are conflicting and concessions have to be made. Also the choice of objectives and cameras are limited, therefore not every desirable configuration is possible. This section describes the different parts of the scanning cycle. Important parameters like sampling density, magnification, and scanning speed will be discussed.

2.4.1 Scanning cycle

Scanning a specimen consists of a number of steps that are repeated until a preset number of cells is analyzed. This is called the scanning cycle. Each field of view (FOV) is focused automatically after which an image is acquired. When the acquisition is finished, the image is processed and the stage is moved to the next FOV.

- Auto-focusing — Automated focusing in fluorescence microscopy is a time consuming and critical step. If auto-focusing fails, the image analysis will fail and the results will not be reliable. To reduce the auto-focusing time it is possible to estimate the focus position of a FOV, based on the previous measured focus positions of other FOV's. This is called the auto-focusing strategy.

- Image acquisition — The image acquisition can be divided into two steps. First the light is integrated on the CCD until enough photoelectrons are accumulated. After the integration, the shutter is closed and the image is transferred to computer memory. How the different images corresponding to the various fluorophores are acquired is determined by the imaging strategy.

- Image analysis — Of course the image analysis depends on the desired application of the system. In our case, automated dot counting in interphase cell nuclei, the analysis consists of finding nuclei in the image, detecting the dots within the nuclei, counting the number of dots per nucleus and then updating the results. A detailed description of the image processing algorithm is given in chapter 4.
• Stage motion — Before the system starts analyzing a slide, the operator has to define a scan area on the slide. This area is scanned following a meander scan.

2.4.2 Imaging strategy
Imaging a specimen that contains multiple fluorescent dyes can be achieved with different configurations. For example, one can use a color camera instead of a monochrome camera or one can use single band-pass filters instead of multiple band-pass filters. Each configuration will have their unique characteristics. This is called the imaging strategy. Three possible imaging strategies are discussed: single monochrome imaging, sequential monochrome imaging, and simultaneous color imaging.

• Single monochrome imaging — This strategy makes use of dual band-pass filter blocks, both emission and excitation, that are suitable for the two dyes being used. The two dyes are acquired simultaneously into a single monochrome image. If the two dyes in combination with the filter block are properly selected, the color differences can be distinguished on the basis of their gray-level differences. In our case, using DAPI counter stain and a Spectrum Orange probe, the dual band-pass filter block maps the blue signal into the middle gray values and the red signal into the light gray values. This is the simplest technique to image two different dyes. This strategy cannot be used with more than two dyes because it generally will not be possible to distinguish more colors based on their gray-level intensity.

• Sequential monochrome imaging — This second strategy does not have that disadvantage. Multiple dyes can be used simultaneously. For each dye a monochrome image is acquired sequentially with the specific single excitation filter in combination with a multiple band-pass emission filter. If the emission spectra of the dyes do not severely overlap, the emitted light of each dye is acquired in a separate color channel. This has the advantage that the different fluorescent signals cannot interfere with each other. There will only be a little cross-talk between the channels. Depending on the type of filters, the transmission (per wavelength) for a single excitation filter can be larger than for a multi-band excitation filter. Increased excitation light will reduce the integration time.

• Simultaneous color imaging — A 3 chip RGB color camera can acquire three images simultaneously. The third strategy is based on such a camera. The different dyes are imaged on the sensor simultaneously by a multiple band-pass filter block, both emission and excitation. The prism block within the camera sorts the image into three channels. If each dye has to be acquired in a separate channel, this strategy is limited to three dyes. The dyes much be chosen in such a way that the emission spectra of the dyes fall within the spectral characteristics of the RGB channels. If the signal of the dyes is spread over
the channels, one can use color compensation (Castleman 1993), to remove the cross-talk spreading of the fluorescent signal among the color channels. Thereby isolating each dye into a single channel. This technique requires that the number of dyes is equal or less than the number of channels. Of course it is possible to distinguish more than three dyes based on their color. This would require that the dyes do not spatially overlap. In other words one pixel only measures the intensity of one dye at a time. If one pixel measures the intensities of two dye or more, there will not be an unique solution to separate the fluorescence signals into the individual channels.

The three imaging strategies have their own advantages and drawbacks. Single monochrome imaging is the most simple strategy. No filter wheel is needed and a monochrome camera can be used. This strategy is limited to two dyes and it is possible that the two dyes interfere with each other. Color information is not available. Sequential monochrome imaging is the most flexible strategy. The number of dyes is not limited and each dye is acquired in its own channel. This strategy requires a filter wheel to change the excitation filter. The acquisition time will be longer because the images are acquired sequentially. This is not the case with simultaneous color imaging. A filter wheel is not required. Instead of a monochrome camera this strategy makes use of a 3 chip color camera. The dyes must be chosen so that they are properly distributed among the three channels. This makes simultaneous color imaging less flexible than sequential monochrome imaging. There is one drawback using a 3 chip color camera. So far, we have not found a camera that allows different integration times for the three channels. If the brightness between the dyes differs too much, one channel will be saturated before another channel has integrated enough signal.

2.4.3 Spatial sampling density
The sampling density is determined by:

\[ f_s = \frac{M_{obj} \cdot M_{rel}}{\Delta} \quad \text{(pixels/μm)} \]  \hspace{1cm} (2.23)

where \( \Delta \) is the pixel spacing of the camera and \( M_{obj} \cdot M_{rel} \) is the overall optical magnification. The combination of these three parameters have to be chosen in such a way that they satisfy the required sampling density. The required sampling density is strongly related to the image processing and image analysis procedures. Specific image processing techniques may require a sampling density above a certain level to achieve the desired accuracy. On the other hand, the sampling density can be limited by the desired scanning speed. The scanning speed \( v_{scan} \) is defined as the total area that is scanned per time interval. The scanning speed is inversely proportional to the square of the sampling density as well as to the integration time. Assuming that the integration time is determined by the amount of photoelectrons that have to be accumulated, the integration
time is inversely proportional to the image brightness, and proportional to the area of one CCD pixel. Finally the scan speed is proportional to the number of pixels of the CCD chip (parallel dimension $P \times$ serial dimension $S$). Combining these results with eqs. 2.2 and 2.23, the scanning speed is given by:

$$v_{\text{scan}} \propto \frac{P \cdot S}{T \cdot f_c} \propto \frac{\Delta^2 \cdot NA^3}{M_{\text{rel}}^4 \cdot M_{\text{obj}}^2} \cdot P \cdot S$$

(2.24)

It is interesting to see that if the sampling density is increased by selecting another objective with the same $NA$ but a different magnification, only the amount of data that has to be acquired will be increased. But if another camera has been selected with a smaller pixel size, the integration time must also be longer. For example using the Photometrics camera ($\Delta = 6.8 \ \mu$m) instead of the Sony camera ($\Delta = 11 \ \mu$m), the integration time must be $(11/6.8)^2$ times longer. Besides a longer integration time, $(11/6.8)^2$ times more data have to be acquired and processed. This typical example shows that it is more efficient to increase the objective magnification than selecting a camera with a smaller pixel size. The difference between the dimension of the two cameras has not been taken into account with this example. The CCD dimension of the Photometrics camera is about 3 times larger than the Sony chip. Using the Photometrics camera instead of the Sony camera to increase the sampling density will increase the scan time by a factor of $(\frac{11}{6.8})^4 \cdot 3 = 2.2$.

From LSI system theory, the sampling frequency should be higher than twice the highest frequency $f_c$ of the analog signal. Then the analog signal can be recovered from the samples by means of a low-pass filter (Oppenheim et al 1983). This theorem is called the sampling theorem and the required sampling frequency is commonly referred to as the Nyquist rate. The microscope system is a band limited system with a cut-off frequency $f_c$ (eq. 2.4). The Nyquist frequency is then given by:

$$f_N = 2 \cdot f_c = \frac{4 \cdot NA}{\lambda}$$

(2.25)

If the image is sampled properly, practical interpolation schemes (Pratt 1991), such as cubic B-spline and cubic convolution, can be used to increase the sampling density digitally, without any significant degradation of the image quality (Netten 1990). Sampling just above the Nyquist frequency is sufficient. Every required sampling frequency can then be obtained by interpolation. Using the Sony camera in combination with $NA = 1.3$ and $\lambda = 615 \ \text{nm}$, the Nyquist frequency becomes $f_N = 8.5 \ \text{pixels/\mu m}$. To sample above the Nyquist rate, a magnification $M > 93$ would be required. This would not be acceptable if a large area has to be scanned. More than 16,000 images have to be acquired to scan 1 cm$^2$. Using the Photometrics camera, 4,000 images still have to be acquired. It is obvious that choosing a low $NA$ objective would reduce the Nyquist rate and a lower sampling density could be used. The scanning speed however, will not be
increased. The effect of a lower sampling density is canceled by a longer integration time. To increase the scanning speed, the sampling density must be chosen below the Nyquist rate. There are arguments that makes it plausible, that a sampling frequency below the Nyquist rate can still result in a good performance.

The Nyquist rate defined by eq. 2.25 is based on the theoretical cut-off frequency of the OTF for a diffraction limited optical system. Experimental results (Lopes Cardozo 1996) showed that the measured cut-off frequency is often below the theoretical cut-off frequency. Figure 2.18 shows the measured OTF of a x60/1.3 objective. The illumination wavelength was $\lambda = 500$ nm. This example shows that the measured OTF is close to zero for a frequency above $0.75 f_c$.

![Figure 2.18: The measured optical transfer function of a 60x/1.4 oil immersion lens.](image)

The sampling theorem defines at which sampling frequency a band limited analog signal can be reconstructed but does not say anything about the accuracy of measurements or the detectability of a FISH dot. For example measuring the area of an object is just counting the number of pixels associated with the object. The accuracy of this estimator is not related to the Nyquist frequency but is related to the number of pixels covered by the object. If we wish to measure the area of a cell with a diameter of 10 $\mu$m and a coefficient of variation $CV < 1\%$ is required, a sampling density of 20 pixels per diameter is sufficient (Van Vliet 1993). The required sampling density $f_s = 2$ pixels/\(\mu\)m will be far below the Nyquist rate of high NA objectives.
The detectability of a FISH dot is strongly related to the contrast between the dot and the background. Piper (Piper 1996) showed that the sampling density can be reduced to half the Nyquist rate before the digitized dot brightness suffers severely. However, a low sampling density will reduce the ability to discriminate between closely adjacent dots. These examples show that it is not always necessary to sample at the Nyquist rate and that a lower sampling density can be used to achieve a desired scanning speed.

2.4.4 Auto-focusing in fluorescence microscopy

Auto-focusing is essential in automated microscope systems. It is a time consuming and critical step. The auto-focus algorithm finds the maximum of a focus function. The focus function is a measure of focus as a function of the axial (z) position and is sampled at different position along the z-axis. A sequence of images has to be acquired to find the focus position. Due to the low light level intensities of fluorescence microscopy the focusing time can be long compared to the total screening time.

![Figure 2.19: The optical transfer function of a microscope system for different degrees of defocusing.](image)

The focus function should be sensitive to small changes in the focus position. To determine a proper focus criterion, the following aspects should be considered: the image formation through a microscope; the image acquisition with a CCD camera; the objects within the image. Boddeke et al. (Boddeke et al. 1994) proposed that a focus criterion should measure the signal energy of the middle frequencies of the OTF, since defocusing mainly reduces the frequencies around half the cut-off frequency of the optical system. Figure 2.19 shows the OTF of a microscope for different degrees of defocusing. It is obvious that near the in-focus position the frequency content of the OTF at half the cut-off frequency is strongly related to the z-axis position. Although defocusing also reduces
Image acquisition

the higher frequencies content, the signal energy is low and the signal-to-noise ratio will be less. If the focus error becomes larger the middle frequencies are reduced to almost zero and only the low frequencies are effected by the focus position.

**Figure 2.20:** A typical image of a cell nucleus with two hybridization dots and the estimated spectrum of that image. The average line spectrum has been estimated by averaging the modulus of the 1D FFT over all rows.

If the focus criterion is based only on the OTF, it is assumed that the objects that are imaged have a white spectrum. A white spectrum means that the power spectrum is constant for all spatial frequencies. In a practical situation this will not be the case. Figure 2.20 shows a typical image of a cell nucleus with two hybridization dots and the estimated Fourier spectrum of that image. The nucleus and the probe are acquired simultaneously into one monochrome image using the Photometrics camera and the 40x/1.3 objective. This results in a spatial sampling frequency $f_s = 5.9$ pixels/μm. The background has been subtracted from the original image. From each image row, the one
dimensional fast Fourier transform (FFT) is calculated (see figure 2.20). Most of the signal is around the zero frequency. When the objects get smaller, like the dots, the spectrum will be wider. The one dimensional spectrum has been estimated by averaging the modulus of the one dimensional FFT over all rows. This result shows that the frequency content is reduced below 1%, if the spatial frequency $f > 1.3 \ \mu\text{m}^{-1}$. The cut-off frequency of the optical system is $f_c = 4.2 \ \mu\text{m}^{-1}$. In this case the focus criteria should not measure the signal power of the middle frequency because there is no signal from the objects. A band-pass filter at a lower frequency should be used.

The auto-focusing algorithm is based on a derivative filter (Boddeke et al 1994). A digital filter $[1, 0, -1]$ is applied in the $x$ direction of the image $i(x, y)$. The "energy" in the resulting image is computed given the focus function:

$$F(z) = \sum_{x,y} (I(x+1,y) - I(x-1,y))^2$$  \hspace{1cm} (2.26)

$F(z)$ is to be maximized over $z$, then the image is in focus. The $[1, 0, -1]$ filter is a band-pass filter with a central frequency of the band $f_{BP} = f/4$. Because the central frequency is a function of the spatial sampling density, it can be adjusted in such a way that the digital filter passes that part of the spectrum that depends most on the focus position. The sampling frequency determines which part of the spectrum will be considered. The middle frequencies of the OTF are selected when the sampling density is equal to the Nyquist rate. The higher frequencies are selected with over sampling whereas the lower frequencies are selected with under sampling. Although under sampling causes aliasing, it still can be a good focus criterion since it does not affect the signal power at the selected frequencies in the analog image.

Binning is used to achieve the desired (under) sampling frequency. Binning can be done on the CCD chip or digitally after the acquisition. Binning has the advantage that it reduces the integration time, the readout time, processing time and increases the SNR. To determine the "optimal" sampling density, the focus function has been measured for different binning factors. The experiment has been done with the Zeiss microscope system and the Photometrics camera using a x40/1.3 objective. The binning factor has been varied from 2x2 to 16x16. The integration time was the same for all binning factors. The total amount of photons acquired in one image has been kept constant. Figure 2.21 shows the results. The error interval has been defined as two times the standard deviation of the focus function. The focus function is very noisy with a low binning factor. Increasing the binning factor reduces the error intervals. On the other hand the peak of the focus function will be less sharp and the maximum will be more difficult to estimate. The range of the focus function increases with a larger binning factor.
Image acquisition

Figure 2.21: The measured focus function for different binning factors. The binning factor has been varied from 2x2 to 16x16. The error interval has been defined as two times the standard deviation of the focus function.
From a practical point of view a large binning factor is preferable because it reduces the auto-focusing time. A 12x12 binning is used with the automated dot counter. The range is then more than 20 μm and the peak is sharp enough to produce an accurate estimation of the focus position. The focus function is first sampled with a focus step size of 0.8 μm to localize the maximum. Around the peak a step size of 0.2 μm is used. The focus position is estimated by the weighted average of the four samples around the peak. To focus one FOV an average of 6 images have to be acquired each with an integration time of 40 msec. each. Due to the slow readout rate of the Photometrics camera, the whole procedure requires about 10 sec. per FOV.

### 2.4.5 Auto-focusing strategy

In spite of the large binning factor, auto-focusing is still a time consuming step. To reduce the auto-focusing time when a slide is scanned, the number of FOV's that has to be focused should be reduced. Two different strategies — extrapolation and interpolation — can be used to estimate the focus position of one field based on the measured focus position of other fields. Figure 2.22 shows the focus positions of a scan area of 10 by 10 FOV's, obtained with the Leitz system and a x25/0.6 objective. All focus points lie approximately on a smooth planar surface. If we can estimate the course of the focus position as function of the xy position it would be possible to calculate the focus position instead of measuring the focus position.

![focus positions on smooth surface](image)

**Figure 2.22:** The focus positions of a scan area of 10 by 10 fields. All focus points lie on a smooth surface.

- **Extrapolation method** — The focus position is estimated by a linear extrapolation using the focus position of the previous analyzed FOV's. To estimated the focus position of a particular field only the three adjacent FOV's are considered. Because the estimation only uses local information, a focusing error of one field will only influence the estimated focus position of adjacent FOV's.
Interpolation method — Before the actual scanning starts, the focus positions of a number of FOV's has to be measured. A linear fit will estimate the surface $z = ax + by + e$ using the measured focus samples. During scanning the result of the linear fit is used to calculate the focus position of each field. The advantage of the interpolation method is that it uses more focus samples to estimate the surface. If the screening time is long the linear fit is not reliable anymore because the position of the focal plane changes slowly in time due to small temperature variations.

![Graph showing average and maximum focus error for extrapolation and interpolation.](image)

**Figure 2.23**: The average focus error and maximum focus error for both auto-focusing strategies. The focus error is defined as the absolute difference between the estimated focus position and the measured focus position. The reduction factor is the ratio between the number of FOV's that have been scanned and the number of FOV's that have been focused.

We have measured the performance of both methods as a function of the reduction factor, which is the ratio between the number of FOV's that have been scanned and the number of FOV's that have been focused. The two methods have been applied to the data given in figure 2.22. The number of focus samples is first reduced and then these focus samples are used to estimate the focus position of the other FOV's. The result of both methods is compared with the measured focus position. Figure 2.23 shows the average focus error and maximum focus error as a function of the reduction factor. The focus error is defined as the absolute difference between the estimated focus position and the measured focus position. The performance of the interpolation method is much better than the performance of the extrapolation method. Only for a reduction factor of 2 is the focus error of the extrapolation method smaller. The error of the interpolation method seems to be independent of the reduction factor. The average focus error is around 0.7 μm even for
a large reduction factor. If the focus error is acceptable for a specific application the auto-focusing time can be reduced significantly.

There is one drawback when such a technique is applied. This is illustrated with another example of the measured focus positions of a scan area (see figure 2.24). Not all the focus samples lie on a smooth surface. Due to some fluorescence material that has not been removed from the slide after rinsing, the auto-focusing algorithm has failed. It focused on the fluorescent material instead of the nuclei. The fluorescence material is not in the same focal plane as the nuclei. If we focus every FOV, the focus error only occurs where there is some fluorescence material. If we are using the interpolation strategy the focus error also effects the estimated focus positions of other FOV's.

![3D diagram of focus positions](image)

*Figure 2.24:* Another example of the focus positions of a scan area of 10 by 10 fields. The auto-focusing algorithm focused on some fluorescent material instead of the nuclei. The focus samples do not lie on a smooth surface.

### 2.4.6 Scanning time analysis

Although the scanning cycle is described as a sequence of steps, the actual implementation of these steps can be parallelized. Figure 2.25 shows a flow diagram of the scanning cycle for single monochrome imaging. After a FOV is focused the image is integrated on the CCD. During integration the previous image is processed and analyzed. When both, integration and image analysis, are finished, the next image is transferred to the computer memory. At the same time the stage is moved to the next FOV. This diagram can easily be extended for sequential monochrome imaging. The principle is basically the same. During integration of a channel the previous image of that channel is analyzed. This assumes that a channel can be analyzed independent from the channels that still have to be acquired. The excitation filter is changed during camera readout.
Given the flow diagram in figure 2.25 the average time to scan one FOV is given by:

\[ t_{\text{FOV}} = t_{\text{focus}} + \text{Max}(t_i, t_{\text{proc}}) + \text{Max}(t_r, t_{\text{move}}) \]  

(2.27)

where Max() is the maximum operator, \( t_{\text{focus}} \), \( t_i \), \( t_{\text{proc}} \), \( t_r \), and \( t_{\text{move}} \) are respectively the average focusing time, the integration time, the image processing time, the readout time and the time to move from one FOV to the next FOV. As an example, we want to use the Zeiss system with a 40x/1.3 objective, and the Photometrics camera to scan a slide. Using the typical values of \( t_{\text{focus}} = 10 \) sec. (each FOV is focused), \( t_i = 1.0 \) sec., \( t_r = 2.6 \) sec. and \( t_{\text{move}} = 0.4 \) sec. the scanning time per FOV for this configuration becomes \( t_{\text{FOV}} = 12.7 + \text{Max}(1.0, t_{\text{proc}}) \). If we assume that the image processing time is shorter than 1.0 sec., the scanning time becomes \( t_{\text{FOV}} = 13.7 \) sec. In this example the scanning time is dominated by the long readout time of the Photometrics camera. Using the Xillix Micro Imager instead of the Photometrics camera the scanning time per FOV could be reduced to 6.0 sec. The Xillix camera has a 16x faster readout rate. Focusing still requires 4.6 sec. per FOV. To reduce the scanning time even more, the number of fields that have to be focused should be reduced. Using the interpolation method with a reduction factor of 10, the scanning time becomes 1.9 sec. In this case the scanning time is limited by the integration time. At this point, a significant improvement of the scanning speed can only be achieved with a shorter integration time. A shorter integration time requires an improvement (increase) in the fluorescence intensity of the specimen. It is interesting to see, that if we optimize the scanning speed of an automated fluorescence microscope system, at the end it will be limited by the number of photons that have to be acquired.

\[ \text{Start scanning} \]

\[ \text{Focus field} \]
\[ \text{FOV}_n \]

\[ \text{Integrate} \]
\[ \text{image} \]
\[ I_n \]

\[ \text{Analyze} \]
\[ \text{image} \]
\[ I_{n-1} \]

\[ \text{Readout} \]
\[ \text{image} \]
\[ I_n \]

\[ \text{Move to next} \]
\[ \text{field} \]
\[ \text{FOV}_{n+1} \]

\[ \text{# nuclei} > N \]
\[ \text{Yes} \]

\[ \text{Stop scanning} \]
\[ \text{No} \]

**Figure 2.25**: A flow diagram of the scan cycle for single monochrome imaging. Where \( n \) is the index of the FOV that is processed. The cycle will be repeated until \( N \) nuclei are analyzed.

### 2.5 Discussion

This chapter describes the basic building blocks of an automated scanning system that is suitable for fluorescence microscopy. Different aspects have to be considered in order to develop a system that will meet the requirements of a particular application. Numerical aperture, magnification, integration time, and sampling density have to be determined
carefully to achieve the desired performance. The performance can be characterized in terms of resolution, depth-of-focus, contrast, SNR, and scanning speed. A large variety of cameras and microscopes in combination with the large number of parameters makes it difficult to define an "optimal" configuration for a scanning system. Because a large number of aspects has to be considered, not every aspect has been discussed in detail. The image acquisition have been described from a practical point of view.

As a typical example of how a scanning system can be configured, we discuss the image acquisition setup that has been used with the automated dot counter. The automation of fluorescent dot counting in interphase cell nuclei requires an accurate and fast system. The image analysis consists of detecting the cells followed by the detection of the dots within the cells. Features are measured for both the cells and dots which are used to verify the results. The following configuration has been used with the automated dot counter: Zeiss microscope system with a x40/1.3 objective, and a dual band-pass filter block (DAPI and Spectrum Orange), the Photometrics camera with 2x2 binning, and an integration time $t_i = 1.0$ sec., single monochrome imaging and each field is focused. We have used the Zeiss system instead of the Leica system because the hardware components are more reliable. Although the Mörzhäuser stage and focus control are slightly faster, the differences with the Ludl control are small compared to the total scanning time. We have used a high NA objective to allow a short integration time. The slides we have used contain lymphocytes from cultured blood. The nuclei appear flat and they are all in the same focal plane. The small depth-of-focus did not cause any problems with these slides. However the thickness of the nuclei is related to preparation technique (Rijke et al 1996). The main disadvantage of the objective is that it is an oil immersion lens. In a clinical setting, where a large set of slides has to be analyzed automatically, this is not desirable. The x40 magnification in combination with 2x2 binning results in a sampling density of $f_s = 2.9$ pixels/μm. This sampling density is at about one third of the theoretical Nyquist rate. The centromeric 8 probe can still be detected properly because a dot is still covered by an average of 20 pixels. The 2x2 binning factor reduces the integration time and the readout time. We have used single monochrome imaging because the results will not improve with sequential monochrome imaging (see Chapter 5). Single monochrome imaging does not require a filter rotor and the acquisition time is shorter. Finally, each field is focused because the accuracy of the system is more important than the scanning speed. Reducing the number of fields that have to be focused can introduce focusing errors. Although each FOV is focused the total screening time to analyze 500 nuclei in about 15 min. A more detailed description of the performance of the dot counter is given in chapter 5.
Chapter 3

A Fast Scanner for Fluorescence Microscopy using a 2-D CCD and Time Delayed Integration

Hans Netten, Lucas J. van Vliet, Frank R. Boddeke, Peter de Jong and Ian T. Young

(Bioimaging, 2, 1994)

We have developed an imaging system for high speed image acquisition in fluorescence microscopy. The use of a two-dimensional CCD array in a special operation mode called TDI (Time Delayed Integration) permits a significant increase in photon integration time compared to 1D scanners (higher signal-to-noise ratio) without compromising the total data throughput rate. Instead of a start-stop system we use continuous stage motion in the CCD’s parallel shift direction. Synchronizing the parallel clock and the stage velocity guarantees a one-to-one relationship between a moving cell and its image onto the CCD. Compared to start-stop systems, TDI scanning offers a speed improvement, negligible blurring in the scanning direction and a complete suppression of pixel variability boosting the SNR more than 10 dB.
3.1 Introduction

Image cytometry – while it offers the opportunity for accurate and precise measurement of cell properties as well as confirmation of detection – has been limited to small sample sizes. In a number of important areas the need for instrumentation for high-speed measurement of cells and cellular constituents has outstripped available systems. In modern molecular cytogenetics, techniques have been developed to selectively label various DNA sequences with fluorescence tags in interphase as well as metaphase cells. This technique is called fluorescence in situ hybridization (FISH) (Carter 1994). FISH makes it possible to detect structural chromosomal abnormalities in metaphase spreads. Structural abnormalities include translocations, amplifications and deletions. FISH also allows large scale screening to detect an abnormal copy number of a specific chromosome in interphase cells. In order to obtain a reliable estimate for the frequency of occurrence a large number of cells needs to be analyzed (Netten et al 1996 b).

A bottleneck in fluorescence image cytometry is the low intensity of fluorescently labeled probes. Often intensified CCD cameras are used to deal with low light levels. But such systems do not have the proper characteristics, because the intensifier causes a boost of the photon shot noise and reduces the spatial resolution. Low intensities require special cameras that are capable of accumulating signals, for tens of seconds. A slow-scan cooled CCD camera has the required characteristics. Such a camera offers a linear photometric response over a large intensity range, cooling that effectively suppresses the dark current to allow long integration times and a slow readout rate to provide a low readout noise.

Traditionally, automated systems capture images field after field. Image acquisition (exposure and readout) and stage movement from one field to the next field takes place during the processing time of the previous image. The stage movement starts directly after the exposure, during readout. Using a slow scan CCD camera the stage motion will be finished before the readout is completed. Real-time image processing* can be achieved when the processing time is shorter then the total time needed for image acquisition and stage movement. Throughout this paper this field approach will be referred to as static image cytometry. In dynamic image cytometry there is a continuous flow of data that is processed and analyzed in real-time. As a consequence, the microscope stage moves non-stop to offer new scenes at the input of the system. Dynamic imaging systems have been developed for bright field microscopy (Stark et al 1989, Tucker et al 1987). These imagers make use of a one dimensional linear CCD and are not suitable for low light intensities. The data throughput will be very low for long integration times. Using a two

* Real-time means a rate such that the previous image is processed before the next image is offered. Sometimes this means video rate
A fast scanner for fluorescence microscopy

dimensional CCD combined with time-delayed-integration a high data throughput is possible without compromising the integration time.

A number of time-delayed-integration imagers have been reported in the literature (Schlig 1986). These scanners are designed to be used for page and document imaging. Special TDI CCD sensors are used to achieve high speed and signal-to-noise ratio even with low light levels (Barbe 1976). The TDI sensors are characterized by the width of the array (serial dimension) and the number of stages (parallel dimension). A typical example of such an array is 2048 x 96 TDI chip. Signal level increases as the number of TDI stages increases whereas the total noise level increases by the square root of the number of stages (Chamberlain and Washkurak 1990).

We have designed a TDI scanner suitable for fluorescent image cytometry. It is built around a slow-scan cooled CCD camera that is normally used for static imaging. This chapter presents the design and performance of such a system. The chapter is organized as follows. In section 3.2 we describe the principle of TDI scanning. Section 3.3 gives a summary of the design parameters. The actual implementation is presented in section 3.4. We have evaluated the scanner by measuring the SNR, spatial frequency response and speed. The experimental results are given in section 3.5. Finally, in section 3.6 we draw some conclusions.

3.2 TDI Scanning principle

A CCD element consists of a two dimensional matrix of square photosensitive sites, the electron wells. This matrix is called the parallel register. The CCD contains a second register, the serial register, which is a one dimensional CCD itself, and plays an important role during CCD readout. In the operation of a full frame CCD we distinguish two stages, the integration time in which the exposure takes place, and the readout time. When sufficient photon-induced electrons have been collected in the various CCD wells, the CCD is ready for readout. During the readout time all charge stored in the parallel register is shifted (in parallel) towards the serial register. Each cycle the parallel clock causes a shift of exactly one row. The charge stored in the top pixel row is shifted from the parallel register into the serial register. Once in the serial register, the serial or pixel clock transfers the charge packets to the amplifier and A/D converter. After the serial register is emptied the whole cycle of a parallel shift followed by serial readout is repeated until the parallel register is emptied (Aikens et al 1990).

TDI scanning combines the light integration with the CCD readout into a continuous process. During integration the continuous movement of the stage is synchronized with
the parallel shift of the CCD. At each parallel clock cycle a new row of pixels at the bottom of the CCD is exposed by a new scene. As the scene moves, the corresponding row also shifts in the same direction at each cycle of the parallel clock. Finally this row reaches the top of the CCD and is shifted into the serial register and then to the A/D converter. Figure 3.1 shows three steps of TDI scanning. Considering the black pixel in the left-most column, the pixel shifts towards the serial register synchronized with the motorized stage. The pixel integrates from the bottom of the CCD until it reaches the serial register. TDI scanning permits continuous movement of the stage (high throughput) without compromising the photon integration time (high SNR). This leads to a continuous pixel stream during the integration. The result is an image of $S \times N$ pixels, where $S$ is the serial dimension of the CCD and $N$ can be extremely long.

![Figure 3.1: Three stages of TDI scanning at intervals separated by 4 cycles of the parallel clock. The numbered black dots are moving upwards and are imaged on a CCD matrix.](image)

### 3.3 TDI Scanning parameters

To avoid unnecessary blurring, both the scanning speed and the scanning direction need to be controlled. TDI scanning requires precise alignment between the parallel shift direction of the CCD and the direction of stage movement. The integration time can be controlled by adjusting the stage velocity and shift frequency. The values for the parallel clock and the stage velocity depend on the camera and the microscope setup. For a
required total integration time $T_i$ (that depends on specimen brightness), the parallel shift frequency $f_p$ is given by:

$$f_p = \frac{P}{T_i} \quad \text{(Hz)}$$  \hspace{1cm} (3.1)

where $P$ is the parallel dimension of the CCD chip. With a frequency $f_p$ a row of pixels shifts from the bottom to the top of the CCD in $T_i$ seconds. As the CCD shifts one pixel the stage must move one pixel distance divided by the magnification. The stage velocity is given by:

$$v_s = \frac{P \cdot \Delta}{T_i \cdot M} = \frac{f_p}{f_s} \quad \text{(μm/s)}$$  \hspace{1cm} (3.2)

where $M$ is the total magnification, $\Delta$ is the pixel size and $f_s$ is the spatial sampling frequency of the microscope system. The spatial sampling frequency can be measured with a bar pattern on a test slide. Using a zoom projection lens, each time the magnification changes the sampling frequency must be measured, therefore a fixed magnification is to be preferred. In our system there is no position feedback from the stage to control the camera shift. Therefore the stage velocity as well as the shift frequency must be very stable and accurate.

The maximum readout rate ($r_{max}$) of the camera limits the shift frequency and stage velocity. Given eq. 3.1, the upper bound of the shift frequency is given by:

$$f_p < \frac{r_{max}}{S} \quad \text{(Hz)}$$  \hspace{1cm} (3.3)

where $S$ is the serial dimension of the CCD. Due to the upper bound of the shift frequency the integration time is limited by a lower bound and is given by:

$$T_i > T_r = \frac{S}{r_{max}} \quad \text{(s)}$$  \hspace{1cm} (3.4)

$T_r$ is the readout time of the camera to acquire a full image. A shorter integration time than the readout time is not possible with TDI scanning. If a shorter integration time is required due to saturation of CCD wells, the emitted light must be reduced. An optical neutral density filter is a common way to reduce the photon flux but, in practice, strong FISH signals are extremely rare.

The scanning speed is defined as the total area that is scanned per second. Each second, $S$ times $f_p$ pixels are acquired. The number of pixels divided by the spatial sampling density squared gives the area that is scanned per second. Combining this result and eq. 3.1 yields the scanning speed:

$$v_{TDI} = \frac{P \cdot S}{T_i \cdot f_s^2} \quad \text{(μm}^2/\text{s})$$  \hspace{1cm} (3.5)
Eq. 3.5 shows that the scanning speed is determined by the required integration time, sampling density and the dimension of the selected CCD. Because of the limitation of the readout rate, the maximum scanning speed is achieved when the integration time is equal to the readout time of the camera. Typical values for all of these variables will be presented in the following section.

Figure 3.2: Schematic overview of a TDI scanning system. The dashed lines separate the camera, microscope and control unit.
3.4 System implementation

3.4.1 Hardware
Our TDI scanning system is built around a Nikon Diaphot epi-illuminated inverted microscope. The microscope is fully automated. Focus, stage, camera and excitation shutter are controlled by a computer. Figure 3.2 shows a schematic diagram of the system. The microscope is equipped with a motorized stage. The stage is driven by two DC motors. Each motor has a position encoder that is part of a closed loop system, controlled by a digital Proportional Integral Derivative filter (PID, model LM 628, National Semiconductors) (Warwick and Rees 1988). One encoder step size is 50 nm. TDI scanning requires a constant velocity of the stage. Our experiments have shown that stepping motors are not suitable for this application. With DC motors the stage velocity is more stable. The PID filter cannot compensate for mechanical disturbance of the stage because the stage is not included in the close loop. The encoder gives the position of the motor and not of the stage. Therefore precise mechanics is used to connect the motors and the stage. Focus control is also driven by a DC motor with a PID filter. Here the encoder step size is 25 nm. A rotation head between camera and microscope is used to align the direction of camera shift and stage motion. The zoom lens can be used to adjust the magnification but has the disadvantage that the sampling density has to be measured each time.

Table 3.1: Specifications of the CCD elements used with the TDI scanner

<table>
<thead>
<tr>
<th>CCD element</th>
<th>Thompson</th>
<th>Kodak</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type</td>
<td>TH 7882</td>
<td>KAF1400</td>
</tr>
<tr>
<td>Element size (S x P)</td>
<td>384 x 576</td>
<td>1317 x 1035</td>
</tr>
<tr>
<td>Pixel size</td>
<td>23 x 23 μm</td>
<td>6.8 x 6.8 μm</td>
</tr>
<tr>
<td>Average (gain⁻¹)</td>
<td>90.9 e⁻/ADU</td>
<td>7.9 e⁻/ADU</td>
</tr>
<tr>
<td>Maximum SNR</td>
<td>56 dB</td>
<td>45 dB</td>
</tr>
<tr>
<td>Operating temperature</td>
<td>−35 °C</td>
<td>−42 °C</td>
</tr>
</tbody>
</table>

A Photometrics 200 series camera (Aikens et al 1990) is connected to the microscope. This is a slow scan cooled CCD camera. A Peltier element cools the CCD below −35°C to suppress dark current. The readout rate is 500 kHz which is slow compared to the 15 MHz of a video camera. An A/D unit converts the collected charge in 12 bits data. The noise level of this camera is photon limited. This means that all other noise sources of the camera are negligible in comparison to the Poisson-distributed photon noise. Photometrics provides different CCD elements for the series 200 camera. We have used two cameras with different elements, the Thompson TH 7882 chip and the Kodak KAF1400 chip. Each chip has its own characteristics. The Kodak chip is very large (1.3 Mpixels) and has small pixels. The Thompson chip is comparable to the size of a CCD
video camera. The specifications are listed in table 3.1. The camera control provides the commands for Time Delayed Integration. The external trigger of the camera synchronizes the parallel shift with the stage motion. The camera control can contain between 4 to 32 MByte memory. The data are temporarily stored in the camera control memory, to await further processing. Camera, stage and focus control are connected to a Sun 4/260 computer with an external VME bus.

3.4.2 TDI scanning in practice

TDI scanning is particularly interesting when large areas have to be scanned. The scanning area is divided into strips. The TDI system images each strip in one scan. Due to the parallel shift of the CCD array there is only one scan direction. After each scan, the stage moves back to the beginning of the next strip.

![Graph](image)

**Figure 3.3:** The measured focus position and the result of a linear fit with three focusing samples of a strip of 3.2 mm. The bulbs are the absolute difference between the measured focus position and the linear fit $z = 8.64 - 2.57 x$.

In the "dead-time" associated with the mechanical "fly-back", the stage stops at a small number of places to focus (Boddeke et al 1994). These samples are used to stay in focus during the subsequent scan. A linear function is fit to the focusing samples. Figure 3.3 shows the result of a fit using three focusing samples. The specimen contained DAPI counter stained interphase cell nuclei with a centromeric 8 probe labeled with FITC
(provided by the Department of Cytometry and Cytochemistry, Leiden University, Leiden). The length of the strip is 3.2 mm. The linear fit \( z = 8.64 - 2.57 x \) defines the focus position during the next scan. The estimated focus position is compared with the measured focus position. The maximum error is 1.1 \( \mu \)m whereas the average absolute error is 0.4 \( \mu \)m. The depth-of-focus of the objective (Nikon 20x/0.75) is 0.36 \( \mu \)m (Longhurst 1967). The average error is slightly larger then the depth-of-focus. Experiments have shown that it does not effect the result of spot counting in interphase nuclei (Netten et al 1996 b), but other applications may require a smaller error and have to use more focusing samples.

One scan involves five steps: 1) The stage accelerates to the required speed. 2) The camera opens the shutter and starts shifting the parallel register. 3) The continuous data stream is stored in camera memory. The data can be processed during the scan. The first field of data has to be removed as the pixels of the first field do not shift through the whole CCD element. 4) Disable the camera trigger to stop the parallel shift at the end of the strip. One image is now acquired. 5) The stage moves back to the starting point of the next strip and collects focusing data. These five steps are repeated until the total area is scanned. For example to scan an area of 5 x 10 mm\(^2\) with the Kodak chip the system has to acquire a number \( N_s \) of scans each of 10 mm length using the following system setup:

\[
\begin{align*}
\text{integration time} & \quad T_i = 3 \text{s,} \\
\text{objective} & \quad \text{Nikon 10x/0.5,} \\
\text{overall magnification} & \quad M = 25, \\
\text{pixel size} & \quad \Delta = 6.8 \mu\text{m,} \\
\text{serial dimension} & \quad S = 1317, \\
\text{parallel dimension} & \quad P = 1035.
\end{align*}
\]

The scanning parameters, using eqs. (3.1, 3.2, 3.5), are then given by:

\[
\begin{align*}
\text{spatial sampling frequency} & \quad f_s = 3.68 \mu\text{m}^{-1}, \\
\text{parallel shift frequency} & \quad f_p = 345 \text{Hz,} \\
\text{stage velocity} & \quad v_s = 93.8 \mu\text{m/s,} \\
\text{scanning speed} & \quad v_{TDI} = 33.5 \times 10^{-3} \text{mm}^2/\text{s.} \\
\text{number of scans} & \quad N_s = (5 \text{ mm}) \cdot f_s / S = 14
\end{align*}
\]

Each scan takes 110 s, not including the focus time. The acceleration time is 0.1 s per strip, the readout time of the first field is 3 s and moving the stage back takes about 0.5 s per strip. The total scan time is 1540 s in which 680 Mpixels of data are acquired at an effective rate of 442 kHz. Of the 1540 s only 14\((3+0.5) = 19\) s or 3.5 \% of the total time is spent on initialization and fly-back.
3.5 Performance

To evaluate the performance of TDI scanning, we compare TDI scanning with normal static scanning. The same system is used for both scanning modes, the Photometrics KAF1400 camera. To compare the static to the dynamic approach we examine the improvement in speed and the image quality. The imaging quality is measured in terms of the signal-to-noise ratio (SNR) and spatial frequency response.

![Graph showing speed-up vs integration time](image)

**Figure 3.4**: The speed increment of TDI scanning compared to static imaging for the KAF1400 chip. The maximum speedup of 2 is achieved when the integration time is equal to the readout time $T_r$.

3.5.1 Speed improvement

One reason to use TDI scanning instead of a start-stop scanning is the speed increment. The speed-up depends on the integration time and the readout time of the camera. The readout time is defined as the time to shift one full field out of the CCD into memory at full rate. Considering eq. 3.4, speed-up ($i$) is given by:

$$i = \frac{T_i + T_r}{\max(T_i, T_r)} = \begin{cases} 1 + \frac{T_r}{T_i} & T_i > T_r \\ 1 + \frac{T_i}{T_r} & T_i > T_r \end{cases} \quad (3.6)$$

The numerator defines the time to grab one image with a static system, assuming that the stage moves to the next field during the readout time. The denominator gives the time to
collect the data of one field using TDI. The initialization and fly-back times are ignored. Due to the maximum readout rate of the camera, the scanning time per field is limited by the readout time. Figure 3.4 shows the speedup using the KAF1400 chip. When the integration time is equal to the readout time, TDI scanning gives a maximum speedup of a factor two. Eq. 3.6 shows that the readout time determines the speed increment for a given integration time. The readout time is equal to the number of pixels divided by the fixed readout rate of 500 kHz for the Photometrics 200 series. The consequence of eq. 3.6 is that one should select a CCD element (number of rows and columns) that has a readout time that is comparable to the required integration time. TDI scanning then gives the maximum speed increment.

![Normalized Power Spectrum](image)

**Figure 3.5:** Spatial frequency response in the scan direction for static imaging and TDI scanning. A step edge is imaged using the KAF1400 chip and a Nikon 20x/0.75 objective. The cut-off frequency of the optical system is 2.7 cycles/μm

### 3.5.2 Spatial frequency response

We compared the spatial frequency response of TDI scanning to the response of a static imaging system. The spatial frequency response (SFR) can be estimated by imaging a step edge from which we can derive the overall frequency response of the combined optical system and imaging system (Mullikin et al. 1994). We used the same camera for static imaging and TDI scanning. Static imaging yields a slightly better spatial frequency response than TDI scanning. The reduction of spatial frequencies has several causes. The continuous motion of the stage versus the discrete shift of the camera causes one-pixel
blur in the scan direction of the resulting image. This would be the ideal case when there is a perfect synchronization between stage and camera. A difference in speed will cause additional blurring of the image. Due to the stage motion, vibration of the specimen will smooth the image. Finally if the motion of the stage is not exactly aligned with the CCD shift direction, the image will be blurred perpendicular to the scan direction. To limit the smoothing to half a pixel, the angle between the parallel shift and stage motion must be smaller than \(0.5/P\) rad, where \(P\) is the parallel dimension of the CCD. Experiments have shown that the degradation of the frequency response perpendicular to the scan direction is not significant. The alignment of the system is very easy to accomplish thanks to the rotation head between the microscope and the camera. Figure 3.5 shows the overall frequency response in the scan direction of TDI scanning and static imaging. TDI scanning causes a small reduction in spatial frequency response. At frequency 1 cycles/\(\mu\)m there is 10 \% reduction in resolution, which is hardly visible. The degradation is comparable with 2 pixels blur. If we multiply the spatial frequency response of static imaging with the Fourier transform of a two-pixel wide uniform filter the result is comparable to the spatial frequency response of TDI scanning.

### 3.5.3 Signal to Noise ratio

Considering the SNR we distinguish two aspects. First the variation between two or more independent images (pixel SNR) (Mullikin et al 1994) and secondly the variation between pixels within a single image (image SNR). The variation between two images with the same illumination is caused by three different sources: readout noise, dark current noise and photon shot noise. This method yields the SNR of an individual pixel illuminated by a stationary light source (pixel SNR). The image SNR is caused by the noise sources listed above in addition to the pixel variation. The pixel variation is caused by a different response of the individual CCD wells to the same amount of light. The SNR is defined as

\[
SNR = 20 \log \left( \frac{\mu}{\sigma} \right) \quad (dB)
\]  

(3.7)

where \(\mu\) is the average intensity and \(\sigma\) is the deviation. The variation between two images (average pixel variance) is defined as

\[
\sigma^2_p = \frac{1}{2} \text{var}(I_1 - I_2)
\]  

(3.8)

where \(I_1\) and \(I_2\) are independent uniformly illuminated images with the same average intensity \(\mu\). The standard deviation between pixels (image variance) is defined as

\[
\sigma^2 = \text{var}(I)
\]  

(3.9)

where \(I\) is a uniformly-illuminated image.
Figure 3.6: The image SNR of TDI and static imaging for the whole dynamic range of light intensities.

Figure 3.7: The pixel SNR of TDI and static imaging for the whole dynamic range of light intensities.
Two uniformly illuminated images were acquired for both TDI and static imaging for a range of intensities. The variations defined in eqs. (3.8 and 3.9) and the corresponding signal-to-noise ratios were calculated. Figure 3.6 and figure 3.7 show the results using the KAF1400 chip. The thick gray line is the ideal SNR of a photon limited camera (a camera in which all other noise sources are negligible compared to the photon shot noise induced by the quantum nature of light) and therefore the upper bound for the measured SNR.

In figure 3.7 we see that the pixel SNR of static and TDI scanning are comparable to the ideal SNR of a the photon limited camera. Other noise sources than photon shot noise are not significant. If there is no variation between the pixels (electron wells) we would expect the same result in figure 3.6 for the image SNR. However, this is not the case. The image SNR is much lower for a static system. Using TDI scanning one pixel in the output image is an average over a whole column of electron wells. Therefor the variation is much less and is mainly determined by photon shot noise. It is possible to correct the pixel variation for static imaging using flat field correction. Flat field correction requires a dark field image (shutter closed) and a blank field image (illuminated image without objects) and must be computed using floating point operations. Therefor it is a time consuming operation. If the correction is done properly the image SNR of static imaging will be comparable to TDI imaging.

![Figure 3.8](image.png) A TDI image of DAPI counter stained cell nuclei. The image size is 360 x 3600 pixels, that is corresponding to an area of 0.154 x 1.54 mm² on the slide. We have used the TH 882 chip and the Nikon 60x/1.4 objective.

### 3.6 Conclusions

We have developed a time-delayed-integration (TDI) CCD scanner suitable for fluorescence microscopy. TDI scanning results in a high data throughput without compromising the image quality. Time delayed integration is achieved by a Photometrics slow scan CCD camera. This camera is available with several different CCD chips. We have evaluated the scanner with two different chips, KAF 1400 and TH 7882. Figure 3.8 shows an example of scanning one strip of a slide using the TH 7882 chip. The scanning speed of TDI scanning is at most twice the speed of static scanning. The required integration time and the readout time of the CCD element determine the speed-up of TDI scanning. The spatial frequency response (SFR) of TDI scanning is comparable
to the SFR of a static imaging system. Due to the discrete pixel shift versus continuous motion and synchronization errors there is a small degradation of the spatial frequency response. The resulting blur in the image is comparable to a smoothing filter of \((2 \times 1)\) pixels and is hardly visible. TDI scanning provides a complete suppression of pixel variability. The image SNR of TDI scanning is more then 10 dB better than the image SNR of static imaging. Figure 3.9 shows the results of static and TDI imaging of the same microscope field. Visually there is hardly any difference.

![TDI image (229x425) vs Static image (229x425)](image)

**Figure 3.9:** An example of a TDI image (a) and a static image (b) of the same microscope field. The images are captured with a Nikon 60x/1.4 objective and a integration time of 1.2 s.

Besides the improved SNR, TDI scanning has the advantage that there is shading in only one direction (perpendicular to the scan direction). Another advantage is that the number of image edges is less than with static imaging. The data throughput is therefore higher because fewer objects touch the image border and no overlap in the scan direction is necessary.
Chapter 4

Fluorescent Dot Counting in Interphase Cell Nuclei

Fluorescence in situ hybridization allows the enumeration of chromosomal abnormalities in interphase cell nuclei. This process is called dot counting. To estimate the distribution of chromosomes per cell, a large number of cells has to be analyzed, particularly when the frequency of aberrant cells is low. Automation of dot counting is desirable because manual counting is tedious, fatiguing, and time consuming. We have developed a completely automated fluorescence microscope system that counts fluorescent hybridization dots for one probe in interphase cell nuclei. This manuscript focuses upon the dot detection procedure. Three different algorithms are presented. The problem of "overlapping" dots and split dots are discussed.
4.1 Introduction

In this chapter we will describe the image analysis procedures that have been developed for the automated dot counter. The labeled chromosomes become visible as colored dots in interphase cell nuclei. The aim of chromosome enumeration is to estimate the frequency distribution of chromosomes per cell. A large number of cells has to be analyzed to achieve an accurate estimation of the distribution. This process is called dot counting.

Dot counting involves two segmentation steps, the detection of the nuclei followed by the detection of the individual dots. The basic concept of both segmentation procedures is straightforward. The original image is first processed to define a mask for each nucleus. A number of features of each object is measured. The features are used to select single nuclei. Nuclei that are not properly segmented, touching nuclei and debris are rejected. To detect the dots the image is segmented again within the mask of the nucleus. Features of a possible dot are measured. These features can be used to verify if a detected dot is a real single hybridization dot, and to detect split dots and overlapping dots. After the dots are detected it is easy to count the number of dots and update the results. A user-interface is included to interactively verify and correct the scoring.

All aspects of the image analysis software will be discussed. The software is part of a completely automated microscope system that counts fluorescent hybridization dots for one probe in interphase cell nuclei. Only two colors can be used — one for the counter stain and one to make the chromosome visible. The image acquisition procedure has been discussed in chapter 2. Two imaging strategies have been used in this system, single monochrome imaging and sequential monochrome imaging. The starting point of this chapter is that the images are stored in computer memory.

4.2 Image analysis

This section describes the image processing algorithms that actually segment the nuclei and dots. The algorithm must be accurate. The number of false positives and false negatives must be as low as possible. On the other hand the algorithm must be fast. For example to analyze 500 nuclei and an average of 5 nuclei per image, more than 70 MBytes of data have to be processed. Our algorithm can be divided into four steps: 1) Find a region that contains a nucleus, 2) Find the nucleus in the region, 3) Find dots in the nucleus, and 4) Count spots and update the dot histogram for the entire microscope slide.
The algorithm is basically the same for both imaging methods, single monochrome imaging and sequential monochrome imaging. The only difference is the input image at each step, as shown in figure 4.1. With single monochrome imaging there is only one image that contains both the counter stain and the probe signal. This image is the input for each step. With sequential monochrome imaging two images are available. The counter stain image is the input of the first two steps that deal with the nucleus segmentation. The probe image will be the input of the third step that deals with the dot counting.

![Flow diagram of the image processing algorithm](image)

**Figure 4.1:** A flow diagram of the image processing algorithm. The input image of a routine depends on the imaging method. With single monochrome imaging, one image contains DAPI + Spectrum orange and is subject to each step. With sequential monochrome imaging the two dyes are separated. The DAPI image is subject to the first two steps and the Spectrum Orange image is subject to the third step.

### 4.2.1 Find region of interest (ROI)

The number of cell nuclei per image depends on the cell density of the specimen and can vary from less than 1 cell per image (low density) up to more than 10 cells per image (high density). Considering the cell density and the average cell area, a typical image consists mainly of background. For example with 8 nuclei in an image and an average nucleus area of 2500 pixels, 94% of the total area is background. The goal of this first step is to find those regions in the image that contain one or more cell nuclei in a relatively short time. These regions are subject to the next steps. Instead of processing the whole image only small parts have to be processed.
To speed-up the algorithm the original image is first sub-sampled by a factor 8. The reduced image is pre-filtered by a uniform filter to suppress noise (Pratt 1991), and a gray level opening (Haralick et al 1987, Pratt 1991) is performed to remove the dots. A shading correction is then applied using (again) a gray-level opening. The resulting image is segmented by an automatically-chosen constant threshold $\theta_{Zack}$ (Zack et al 1976). We used this technique because it is suitable for images that contain only a few objects and have a large background area. An enclosing rectangle for each object in the segmented images defines the region of interest.

4.2.2 Cell detection

For each ROI the original image is processed again at full resolution to define a mask for each nucleus. A ROI is first re-segmented into background and objects followed by classification of the objects into real single nucleus, debris, or touching nuclei. A gray-value opening is applied on the original data to remove the spots. The spots can influence the threshold level calculated in the next step. The iso-data thresholding algorithm $\theta_{iso}$ (Ridler and Calvard 1978) is used to segment the ROI into objects and background. We have used $\theta_{iso}$ instead $\theta_{Zack}$ because now we know that there is an object in the ROI and the background area is small. $\theta_{iso}$ is suitable for images that have a gray value histogram with two equal peaks, one of the object and one of the background. The resulting object mask is then further processed using a number of morphological operations to improve the segmentation: Dilation of the objects is used to slightly enlarge the objects to ensure that the dots are inside the object mask. The enlarged objects are eroded and then again propagated to the original objects to remove small objects and noise. Finally a binary opening of the resulting object mask is used to separate slightly touching nuclei. A discussion of these operators can be found in (Haralick et al 1987, Serra 1982).

After the segmentation, a number of features are measured for each object. The features are used to select single nuclei. The following features are computed: size (area, perimeter), shape (contour ratio, sphericity, eccentricity) and intensity (total fluorescent intensity, average intensity) (Young and Roos 1988). Not all features have to be used to select the nuclei. With the experiments described in the next chapter, nuclei are selected based on the area, contour ratio, sphericity, and eccentricity. A simple strategy is used to classify the objects. Results will show that this simple technique is sufficient. Each feature is mapped according to a trapezoidal merit function, which normalizes to a value between 0 and 100. The overall merit is the minimum of all merits calculated for each feature. If the merit is below a certain number the object is rejected. This technique is also used in other automated scanning systems, for example a metaphase finder (Vrolijk 1993). The trapezoidal mapping function is determined on the basis of the visual classification of a limited number of data used as training set.
4.2.3 Dot detection

To count the dots, the original image is segmented again within the mask of the nucleus. Three different techniques are presented. The first algorithm is the simplest one and is based on a Tophat transform followed by constant threshold. This technique is called Tophat Threshold. The second technique is called Laplacian Threshold (nL Threshold) and is an extended version of the Tophat Threshold. After the Tophat transform a Laplacian (nL) filter is applied to separate touching dots. The third technique is again based on the Tophat transform but instead of a constant threshold a variable threshold is used to label the dots. This third technique is named Dot Label.

![Image](image_url)

**Figure 4.2**: An example of Tophat Threshold and nL Threshold. a) Original image. b) Intensity plot of a line crossing the two dots. c) Tophat transform, Tophat[Im] = Im - Max[Min[Im]]. d) Laplacian, nL[Tophat] = Max[Tophat] + Min[Tophat] - 2*Tophat. The index of the Max/Min operator defines the size of the kernel.
4.2.4 Tophat Threshold algorithm

A Tophat transform (Meyer 1979) is performed on the original image to remove the DAPI counter stain. For light objects, on a darker background, the Tophat transform is given by

$$Tophat(A, B) = A - \max_B \left( \min_A \right)$$  \hspace{1cm} (4.1)

where $\max()$ and $\min()$ are maximum and minimum filters, respectively, over a region $B$. We typically choose $B$ to be a 5x5 window. This is illustrated in figure 4.2. The Tophat transform is only applied within the mask of the nucleus. The resulting image only contains the Spectrum Orange dots on a noisy background. The size of the Tophat kernel must be slightly larger than the dots. A constant threshold is performed on the Tophat transform to find the dots. The threshold level is given by $\Theta_{th} = \mu_{bkg} + k \cdot \sigma_{bkg}$ where $\mu_{bkg}$ and $\sigma_{bkg}$ are the mean and standard deviation of the background inside the mask of the nucleus. The mean and standard deviation are estimated using the pixels below the 90% percentile intensity of the Tophat image. The parameter $k$ has to be determined on the basis of a limited number of nuclei used as a training set.

4.2.5 nL Threshold algorithm

Most dots are detected properly with the Tophat Threshold, but some dots appear merged. Therefore, an extra step is included after the Tophat Threshold. A nonlinear Laplacian (Van Vliet et al 1989) is performed on the Tophat image. This step is only applied within the mask of the Tophat Threshold. A threshold on a negative level will separate touching dots (see figure 4.2). The threshold level $\Theta_{NL}$ is determined by half the minimum intensity of the Laplacian image. The mask of the Tophat transform and nL are combined into a mask of detected dots.

![Image](image_url)

**Figure 4.3**: An example of the definition of connectivity. The light gray pixels are already labeled with 1 or 2. The dark gray pixels have an intensity within the threshold-band. Pixel $a$ will be assigned to dot 1. Pixel $b$ will create a new dot if the intensity is equal to $\theta_{seed}$. 
4.2.6 Dot Label algorithm
The Tophat Threshold has the disadvantage that it is difficult to define a proper threshold level. Figure 4.2 shows that if the threshold level is too high a dot will be missed, and if it is too low, two dots will be merged. The nL filter is used to separate touching dots but has the disadvantage that it is sensitive to noise. False dots could be the result. To overcome these problems a different approach is presented.

Again a Tophat transform is performed on the original image. In contrast with the Tophat Threshold, the Dot Label algorithm uses a variable threshold level. The basic idea of the algorithm is that pixels with an intensity that is equal to a threshold level are assigned to a dot if they are connected to that dot. If they are not connected to an existing dot, a new dot is created. The threshold level $\Theta_{seed}$ starts at the maximum intensity of the image $I_{max}$ and runs down until it is just above the background level $\Theta_{seed} = \mu_{bkg} + k*\sigma_{bkg}$. A second threshold level $\Theta_{conn}$ is introduced to avoid false dots. Small variations in the image due to noise, can create false dots. Instead of a threshold level, a threshold-band is used. Pixels within a threshold-band ($\Theta_{seed} \geq I(x,y) > \Theta_{conn}$) are assigned to a dot if they are connected to that dot. Pixels that are equal to the threshold level $\Theta_{seed}$ and not connected to a dot will create a new dot. The width of the threshold-band is related to the SNR of the images, specifically the $\sigma$ associated with the noise. The second threshold level is defined as $\Theta_{conn} = \Theta_{seed} - 3*\sigma_{camera}$, where $\sigma_{camera}$ is the standard deviation of the camera at the intensity $\Theta_{seed}$.

![Diagram](image-url)

**Figure 4.4:** Three steps of the Dot Label algorithm. $\Theta_{seed}$ starts at the maximum intensity of the image. Dot 2 is created when $\Theta_{seed}$ is equal to the maximum intensity of that dot. The algorithm stops when $\Theta_{seed}$ is just above the background level.
A pixel is connected to a dot if there is a path from that pixel to one pixel of the dot, considering all the pixels within the threshold-band. An example is given in figure 4.3. Two dots have been created and the pixels are labeled with 1 or 2. The dark gray pixels have an intensity within the threshold-band. Considering pixel $a$, there is a path, in a 4-connected neighborhood, along the gray pixels to a pixel of dot 1. So this pixel would be labeled as 1. Considering pixel $b$, there is no path to one of the dots. If the intensity of pixel $b$ is greater than or equal to $\Theta_{seed}$ then this pixel is the seed of a new dot with label 3. Figure 4.4 shows three intermediate steps of the algorithm. The threshold level $\Theta_{seed}$ starts at the maximum intensity of the image. This pixel is the seed of the first dot. Pixels within the threshold-band are labeled if they are connected to that dot. The second dot is created when $\Theta_{seed}$ is equal to the maximum intensity of that dot. The threshold level $\Theta_{seed}$ decreases until it is just above the background level.

### 4.3 Feature based dot detection

To refine the result of the dot detection algorithm a number of features are measured. These features can be used to verify if a detected dot is a real hybridization dot, to detect split dots, or to distinguish "overlapping" dots from single dots. The problem of overlapping dots is discussed in the next section. This section presents a number of features that can be measured and how they can be used to improve the result.

#### 4.3.1 Feature extraction

Often features are based on the resulting mask of the segmentation procedure. For example the area is commonly estimated by counting the number of pixels in the mask. Because the dots are relatively small (area = 11 pixels), the area strongly depends on the threshold level. To make the features independent of the segmentation procedure, the measurements are not based on the dot mask. The features are measured using the pixels that have an intensity larger than a fraction $\gamma$ of the maximum intensity of the dot. The maximum intensity $I_{\text{max}}$ of a dot is the maximum intensity within the dot mask. As an example, the total intensity is defined as the sum of the intensities that are larger than $\gamma I_{\text{max}}$ and is given by:

$$I_{\text{tot}} = \sum_{x,y \in \text{dot mask}} I(x, y) \cdot \text{clip}(I(x, y) - \gamma \cdot I_{\text{max}})$$  \hspace{1cm} (4.2)

where

$$\text{clip}(q) = \begin{cases} 1 & q \geq 0 \\ 0 & q < 0 \end{cases}$$

and the set $(x,y)$ is chosen within the dot mask. The fraction $\gamma$ is an arbitrary value between 0 and 1. If $\gamma$ is close to zero, background noise will influence the measurements.
Dot counting in interphase cell nuclei

If $\gamma$ is too high the measurements will be based on only a few pixels. We have used a value $\gamma = 0.33$. The input image $I(x,y)$ is assumed to be corrected for background variation. The Tophat image is used as the input image. The Tophat transform subtracts the estimated background signal from the original image. The following features are measured: maximum intensity, area, total intensity, average intensity, relative intensity, and eccentricity.

- **Maximum intensity** — $I_{\text{max}}$ is the maximum intensity within the dot mask.
- **Area** — $A_{\text{dot}}$ is the number of pixels with $I(x,y) > \gamma I_{\text{max}}$.
- **Total intensity** — $I_{\text{tot}}$ is the sum of the intensities with $I(x,y) > \gamma I_{\text{max}}$.
- **Average intensity** — $I_{\text{avg}}$ is the total intensity divide by the area.
- **Relative intensity** — $I_{\text{rel}}$ is the total intensity relative to the maximum total intensity within a nucleus and is defined as:

$$I_{\text{rel}}(d_i) = \frac{I_{\text{tot}}(d_i)}{\max(I_{\text{tot}}(d_1), \ldots, I_{\text{tot}}(d_n))}$$  (4.3)

where $I_{\text{tot}}(d_i)$ is the total intensity of dot $i$. The relative intensity of the dot with the maximum total intensity will always be one.

- **Eccentricity** — The definition for the eccentricity is:

$$E_{\text{dot}} = \sqrt{\frac{\eta_{20}}{\eta_{02}}}$$  (4.4)

where $\eta_{20}$ and $\eta_{02}$ are the invariant second order normalized moments (Gonzales and Woods 1990). For a circular dot with a brightness distribution that is circularly symmetric, the eccentricity will be one.

**4.3.2 Dot verification**

In principal all features can be used to verify if a detected dot is a real hybridization. To get a better insight in which feature should be used, the expected accuracy of the different features is discussed. There are several factors that can influence the measurements. The number of pixels per dot, which is related to the sample density, affects the accuracy of the feature estimators. A number of publications (Van Vliet 1993, Young 1988) have reported on the relationship between the sample density and the coefficients of variation (CV) of several estimators. Considering the area, the theory predicts a percentage error below 10% (Young 1988), even though the dots are relatively small. However, the measured CV of the area is 35% (the percentage error and the CV are roughly equivalent measures). Thus the variation caused by a limited number of pixels is not a complete explanation of the difference between theory and experiments. A second source that can affect the measurements is the image brightness noise. In our case using a Photometrics camera and an average dot intensity above 500 ADU (camera range is 4095 ADU) the
SNR will be about 30 dB (see chapter 2). Again a variation in the intensity of less than 4% due to noise is not significant in comparison to the measured variation. CV's of 21% and 42% for the intra and inter nuclear distribution, respectively, of the total intensity have been reported (Nederlof et al 1992 a). The non-uniform illumination of the fluorescence microscope is another source that can contribute to the large variation of the intensity features. We have measured a shading of 20%. The shading is defined as the \( (I_{\text{max}} - I_{\text{min}})/I_{\text{avg}} \) of a homogenous FOV. Finally the effect of the focus position of a dot must be considered.

![Graph showing the relative error for five different features as a function of the focus position.](image)

**Figure 4.5:** The relative error for five different features as a function of the focus position. Each data point is an average of the relative error measured for 12 dots. At \( z = 0 \) the dots are in focus. The error intervals of each plotted point are not included because they are significantly smaller than the estimated relative error.

Because nuclei are not flat, the dots are not always in the same focal plane, and it is possible that a dot is out-of-focus. It is also possible that the auto-focusing routine fails because it focuses on debris instead of the dots. Figure 4.5 shows the relative error for each feature as a function of the focus position. The relative intensity is not included. The features are measured at different focus positions. The relative error of a feature \( f \) for a dot at position \( z \) is defined as:

\[
\epsilon_f(z) = \left| \frac{f(z_0) - f(z)}{f(z_0)} \right|
\]

(4.5)

where \( z_0 \) is the in-focus position of the dot. The average relative error for 12 dots is plotted in figure 4.5. All features strongly depend on the focus position. A focusing error
Dot counting in interphase cell nuclei

of 0.5 μm yields a relative error of more than 15% for the area, average intensity and maximum intensity. Only the total intensity and the eccentricity have a relative error less than 10% if the focusing error is on the order of 1 μm.

Each source that influences the measurements will contribute to the large variation of the features. We have measured CV’s varying from 22% for the relative intensity to 51% for the average intensity. The total intensity and the relative intensity are used to verify if a detected dot is a real hybridization dot. If the value of a feature is not within a certain interval the detected dot will be rejected. The interval is defined from the minimum value to the maximum value of that feature as observed in a training set. The training set contains only hybridization dots and no false dots.

![Image](image.png)

Figure 4.6: A gallery of images of cells that contain split dots.

4.3.3 Split dot classification

As a result of cell replication, one target chromosome can appear as two dots that are close together. This is called a split dot. Those two dots should be counted as one dot. Mis-interpretation of a split dot can lead to an apparent high percentage of trisomy cells
in what is actually a normal specimen. Figure 4.6 shows a gallery of cell nuclei that contain split dots. All these cells should be interpreted as cells containing two dots. The two dots of a split dot are always close together and the intensities of both dots are in general weaker than the third dot. The relative intensity is used to detect split dots. Two dots \( d_i \) and \( d_j \) will be classified as a split dot if the following rule is true:

\[
D(d_i, d_j) < D_{\text{split}} \quad \text{and} \quad \bar{I}_{\text{rel}}(..., d_i + d_j, ...) > \bar{I}_{\text{rel}}(..., d_i, d_j, ...)
\]  

(4.6)

where \( D(d_i, d_j) \) is the projected distance between the two dots \((d_i\) and \(d_j))\) and \( \bar{I}_{\text{rel}} \) is the average relative intensity within a nucleus. Two dots are combined \((d_i+d_j)\) if the average relative intensity is larger than if they are not combined \((d_i, d_j)\). In other words the relative intensity of the combined dots must be closer to one. This rule is only applied to a cell with more than two detected dots.

### 4.4 Overlapping FISH dots in 2D images

It is important to realize that we are observing a three-dimensional nucleus through a two-dimensional projection, and thus one dot can hide "behind" another dot. Normal interphase cell nuclei contain two copies of all autosomes. Microscope imaging projects the three dimensional nucleus onto a two dimensional sensor. Two dots can be distinguished if the projected dots do not severely overlap. "Overlapping" dots means in this thesis that the projected distance between two dots is too small to separate the dots properly.

#### 4.4.1 The probability of overlapping dots

We have calculated the probability that in a nucleus with two dots the projected dots overlap by modeling the nucleus as an oblate spheroid. Cell nuclei in suspension have a spherical shape. In the process of slide preparation they turn into bodies of revolution called oblate spheroids, i.e. ellipsoids with principal axes \( R_c, R_c, R_h \) where \( R_c \) is the radius of a nucleus in the xy-plane and \( R_h \) is the principal axis parallel to the z-axis. Given an oblate spheroid, the height \( h(x,y) \) of the body in the xy-plane is then given by:

\[
h(x,y) = 2 \cdot R_h \sqrt{1 - \left(\frac{x^2 + y^2}{R_c^2}\right)}
\]

(4.7)

If the position \((x, y, z)\) of a dot is equally likely to be anywhere within the volume of the nucleus, the probability density function to find a dot inside an oblate spheroid at position \((x, y)\) is:

\[
p(x, y) = \frac{h(x, y)}{\frac{4}{3} \pi \cdot R_e^2 \cdot R_h} = \frac{3 \sqrt{1 - \left(\frac{x^2 + y^2}{R_c^2}\right)}}{2 \pi \cdot R_e^2}
\]

(4.8)
where the denominator is the volume of an oblate spheroid. The two dots overlap when the second dot lies inside a cylinder with radius \( r_o \) that is centered at the \( x, y \) position of the first dot. This is illustrated in figure 4.7.

![Diagram of overlapping dots](image)

**Figure 4.7:** Two "overlapping" dots when we are observing a three-dimensional nucleus through a two-dimensional projection. The two dots overlap when the second dot lies inside a cylinder with radius \( r_o \) that is centered at the \( x, y \) position of the first dot.

The radius \( r_o \) is the smallest distance between two projected dots that can still be separated. The probability of an overlap is then given by:

\[
P_o(D(d_1,d_2) < r_o) = \int_{x,y} \rho(x,y)(p(x,y) \cdot \pi \cdot r_o^2)dx\,dy = \frac{3}{8} \left( \frac{r_o}{R} \right)^2
\]  

(4.9)

with \( D(d_1,d_2) \) the lateral distance between the two dots. The above calculation assumes that: 1) The probability density function of the position \( (x, y, z) \) of a dot inside a nucleus is uniformly distributed; 2) the positions of the dots are independent; 3) \( r_o<<R_c \).

Frequency distribution curves of observed distances between two targets have been compared with a model that assumes uniform and independent distribution of point-like targets. There is evidence that chromosomes occupy distinct territories in cell nuclei. Dietzel et al. have shown a significant difference between observed data and this model (Dietzel et al 1995). But the differences are small, especially when the distance between the targets is small. Although the above assumptions are not completely correct, eq. 4.9 yields a good approximation of the probability of an overlap.

It is interesting to see that the probability of an overlap is independent of the spheroid's eccentricity and is inversely proportional to the projected area of the nucleus. In other
words, flattening the nuclei on a slide, which increases the area, will reduce the probability of an overlap.

The distance \( r_o \) depends on the radius of an observed dot \( R_d \) and on the capability of the image processing algorithm to separate the dots. \( R_d \) is determined by the physical size of a dot and the point-spread-function of the optical system which depends on the NA of the objective and the emission wavelength \( \lambda \) of the fluorescent dye. If the physical dots are smaller than the wavelength of light, each observed dot is approximately an Airy disc with \( R_d = 0.61 \cdot \lambda / NA \). The probability of an overlap becomes:

\[
P = 0.42(\alpha \cdot \lambda / NA \cdot R_c)^2
\]

(4.10)

where \( \alpha \) is a parameter that represents our ability to segment two adjacent dots. In this case \( r_o \) is defined as \( r_o = \alpha \cdot R_d \). If \( \alpha = 1 \), \( r_o \) is equal to the Rayleigh criterion. The Rayleigh criterion defines a distance at which two superimposed Bessel functions can still be separated based on the maximum intensity. As an example for \( \alpha = 1 \), \( \lambda = 0.615 \mu m \) (peak emission spectrum), \( NA = 1.3 \), and \( R_c = 5 \mu m \), the probability of an overlap \( P = 0.4 \% \). In our case, using a centromeric probe, the radius of an observed dot is generally larger than the radius of an Airy disk. The physical size of a dot is significantly larger than the wavelength of light. In this case, the radius \( r_o \) has to be determined experimentally.

4.4.2 Detection of overlapping dots

The problem of "overlapping" dots is especially important for the detection of a monosomy. Monosomy means that a proportion of the nuclei has only one copy of the target chromosome instead of two. "Overlapping" dots mainly affect the estimated proportion of cells that contain one dot. In practice most cells have two copies of a chromosome and only a small sub-population of cells have an aberrant number of chromosomes. Due to overlapping, some of the cells with two chromosomes are counted as one. Because the proportion of cells with two dots is much larger than the proportion with one dot, the error will be significant for the estimated proportion of cells with one dot. Although overlapping also occurs with cells with three dots, the effect on the estimated proportion of cells with two dots is much smaller. The proportion of cells with three dots is in practice much smaller than those with two dots.

To improve the results of the image processing algorithm we want to include an extra step that classifies detected dots into single dots or "overlapping" dots based on these features. We may expect that two "overlapping" dots will have twice the total intensity of a single dot and that the eccentricity of two touching dots is larger then the eccentricity of a single dot. A nearest neighbor classifier (Fukunaga 1990) has been used to see if it is
possible to discriminate "overlapping" dots from single dots based on the total intensity and eccentricity. The classification is only applied to the cells with one detected dot.

4.5 User interface

The whole system is embedded in a "user-friendly" user-interface to control the microscope, to adjust parameters, and to evaluate the results. This is an essential part of the system. It allows us to make a comparison based on the individual cells, between manual counting behind a microscope, manual counting behind a monitor display, and fully automated counting.

Figure 4.8: An example of a dot histogram. It is possible to relocate nuclei under the microscope and interactively correct the dot counts.

Before the system starts screening, an operator has to define a scan area on the slide. The area is scanned following a meander pattern. After a preset number of nuclei have been analyzed the system stops. The output of dot counting in interphase nuclei can be a dot histogram, or a gallery of images of every nucleus. The output can be used to interactively verify and correct the scoring. In this interactive mode, performed after the scanning cycle is completed, the individual nuclei are relocated automatically under the
microscope. Visual inspection can be done using the monitor display or through the microscope. The user can review and correct the data in a rapid manner through the use of galleries. The galleries can be sorted by dot number, cell features, or dot features. For example, if we want correct the automated scoring of the dot counter we like to review the nuclei with a gallery that is sorted by dot number. Experimental results will show that most of the errors occur with cell nuclei that have been counted as one (focusing errors and overlapping dots) and as three (mis-interpretation of split dots). A gallery sorted by dot number makes it easier to correct these errors. Afterwards a confusion matrix shows the relation between the automated scoring and the interactively corrected scoring. Screen snapshots in figures 4.8, 4.9, and 4.10 show the image gallery sorted by dot number, a dot histogram, and the microscope control.

![Figure 4.9: An example of a gallery of cell images. The gallery is sorted by dot number.](image-url)
Figure 4.10: The microscope control dialog box. Stage, focus, shutter, and filters can be controlled using this dialog box.

4.6 Summary

We have developed an image analysis procedure that counts hybridization dots of one probe in interphase cell nuclei. The algorithm is fast. To find the ROI's the image is subsampled by a factor of 8 which reduces the amount of data by a factor of 8x8. After this first step only a small part of the image has to be processed at full resolution. The segmentation of both the nuclei and dots is based on simple intensity thresholding methods. In addition all two dimensional filters are based on square kernels and can be separated into one dimensional filters which allow a fast implementation.

A critical part of the system is the dot detection algorithm. Three different algorithms are presented. The Tophat Threshold is the simplest one but has the disadvantage that "overlapping dots" can be merged. The nL Threshold algorithm is an extended version of the Tophat Threshold. A Laplacian filter is applied on the Tophat image to separate touching dots. Because the Laplacian filter is sensitive to noise, a third algorithm has been developed, called Dot Label. The Dot Label algorithm is based on a variable threshold level.
After the dot detection algorithm a number of dot features are measured. These features are used in three different classification procedures to verify and improve the result of the dot detection algorithm.

1) Dot verification — The total intensity and relative intensity are used to verify if a detected dot is a real hybridization dot.

2) Split dot detection — A simple rule, based on the relative intensities of the dots and the distances between the dots within a nucleus, is used to detect split dots. This rule is only applied to cells with more than two dots.

3) "Overlapping" dots detection — The eccentricity and total intensity are used to classify a detected dot into a single dot or two "overlapping" dots. We only apply this rule to cells with one detected dot.

The disadvantage of this feature based approach is the large variation of the features. The large variation makes it difficult to discriminate between the different classes. Focusing errors, a low sample density, and non-uniform illumination will contribute to the large variation of the features. Although it is possible to reduce the effect of the different sources, it will always increase the scanning time. For example, increasing the sample density by a factor of two, will increase the scanning time at least with a factor of four. Flat field correction will eliminate the effect of a non-uniform illumination but requires the storage of two calibration images and the calculations have to be done with floating point operations which is time consuming.
Chapter 5

Evaluation of automated FISH dot counting

The automated dot counter has been tested on a number of normal specimens where DAPI was used for the nucleus counter stain and a centromeric probe was used to mark the desired chromosome. The slides contained lymphocytes from cultured blood. We have compared the results of the dot counter with manual counting. Manual obtained results, published in literature, have been used as the "ground truth". For a normal specimen, 97.5% of the cells will have two dots. The machine accuracies, after interactive correction, are comparable to panels of human experts (manual). The fully automatically obtained results are biased with respect to manual counting. An error analysis is given and different causes are discussed.
5.1 Introduction

The performance of the automated dot counter has been evaluated. This chapter describes the different experiments that have been done. The experiments can be divided into two parts. The first part gives the overall performance of the system and different error sources are discussed. The second part focuses on the performance of the different dot detection algorithms.

5.1.1 Scanning procedure

A complete description of the scanning procedure is given in chapter 2. For the sake of clearness the acquisition setup that is used with the dot counter is summarized. We have used the Zeiss microscope system together with the Photometrics camera to acquire the images. With most of the experiments a 40x/1.3 objective has been used in combination with 2x2 binning. This results in a spatial sampling frequency of 2.9 pixels/μm. Single monochrome imaging is applied and each field is focused. Only when a different configuration has been used this will be noticed otherwise the above described configuration has been used.

5.1.2 Biological material

The automated dot counter has been tested on a number of slides where DAPI was used for the nucleus counter stain and a centromeric probe (CEP 8 and CEP 12, Vysis, Downers Grove, IL, USA) was used to mark chromosome 8 or chromosome 12. The probe was labeled with Spectrum Orange. The slides contained lymphocytes from cultured blood. All slides were from normal specimens. One slide was used to train the system and independent slides were used to test the system. The performance of the complete system has been evaluated in a number of experiments with these slides. To evaluate the performance of the different image analysis algorithms a set of images has been used. Two slides have been scanned automatically. From each FOV an image has been acquired and has been saved on computer disk. Afterwards the images have been checked manually to see if they were properly focused. Because the purpose of these experiments was to investigate the performance of the image analysis algorithms, focusing errors have been excluded. A total of 352 images have been acquired, containing 1014 nuclei. A subset of 113 images with 200 nuclei has been used as an independent training set to adjust the parameters of the algorithms. The other 239 images have been used to test the system.

5.1.3 Evaluation method

The result of the dot counter is a dot distribution that gives the proportion of cells containing 0, 1, 2, 3 or > 4 dots. The proportion $p_i$ is estimated by
Evaluation of automated FISH dot counting

\[ p_i = \frac{n_i}{N} \]  
(5.1)

where \( n_i \) is the number of cells with \( i \) dots and \( N \) is the total number of cells. Assuming that the probabilities of the counting errors are constant and the selection of the cells is random, the proportion \( p_i \) is a multinomial distribution. The standard deviation \( s_i \) is then given by:

\[ s_i^2 = \frac{p_i(1-p_i)}{N} \]  
(5.2)

For large \( N \) the multinomial distribution is approximately Gaussian. In that case a 95% confidence interval of the estimated proportion is approximately between \( p_i \pm 1.96s_i \) (Castleman and White 1995). The standard deviation \( s_i \) can be seen as a lower bound for the standard deviation of the estimated probability. For example with a normal specimen we expect that 97.5% of the cells have two dots and if we count 500 nuclei the standard deviation of the measured probability will be at least 0.7%.

5.2 Fully automated screening

To measure the overall performance of the whole system 13 slides have been analyzed with the automated dot counter. The results are compared with manual counting and an error analysis is given. We have used the nL Threshold algorithm to obtain these results.

Table 5.1: Percentage of cells containing various number of dots for different samples as identified manually, with full automation, and with full automation followed by interactive correction.

<table>
<thead>
<tr>
<th>Chromosome 8</th>
<th>N</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>&gt;3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Manual</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spectrum Orange™</td>
<td>800</td>
<td>0.3%</td>
<td>1.5%</td>
<td>97.5%</td>
<td>0.4%</td>
<td>0.0%</td>
</tr>
<tr>
<td>FITC-based Dye</td>
<td>2000</td>
<td>0.7%</td>
<td>2.6%</td>
<td>96.0%</td>
<td>0.7%</td>
<td>0.0%</td>
</tr>
<tr>
<td>Automated</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Slide #1</td>
<td>500</td>
<td>1.8%</td>
<td>2.8%</td>
<td>93.0%</td>
<td>2.2%</td>
<td>0.2%</td>
</tr>
<tr>
<td>Slide #2</td>
<td>501</td>
<td>6.4%</td>
<td>3.8%</td>
<td>84.2%</td>
<td>4.4%</td>
<td>1.2%</td>
</tr>
<tr>
<td>Slide #3</td>
<td>666</td>
<td>2.6%</td>
<td>7.2%</td>
<td>89.3%</td>
<td>0.9%</td>
<td>0.0%</td>
</tr>
<tr>
<td>Automated + Correction</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Slide #1</td>
<td>500</td>
<td>0.6%</td>
<td>0.8%</td>
<td>98.0%</td>
<td>0.6%</td>
<td>0.0%</td>
</tr>
<tr>
<td>Slide #2</td>
<td>501</td>
<td>0.0%</td>
<td>1.3%</td>
<td>97.8%</td>
<td>0.9%</td>
<td>0.0%</td>
</tr>
<tr>
<td>Slide #3</td>
<td>666</td>
<td>3.0%</td>
<td>2.7%</td>
<td>93.8%</td>
<td>0.5%</td>
<td>0.0%</td>
</tr>
</tbody>
</table>

5.2.1 Counting results of normal specimens

The dot distribution can be obtained manually (Manual), fully automated (Automated) and fully automated followed by interactive correction (Automated + Correction). The
first part in table 5.1 shows the dot distribution for normal specimens that is presented by (Kibbelaar et al 1993, Vysis). These manual results will be used as the "ground truth". The second part shows the results of three slides of the dot counter without any interaction of the user. The last part gives the result after correcting the dot counts using the cell gallery on the monitor display. The automatically obtained results can be significantly improved by interactive correction, to the point that they are equivalent to a panel of experts (Manual). An average of about 11% of the nuclei have to be corrected.

Table 5.2: The standard deviation of the dot distribution $P_i$ for three bins $i = \{1, 2, 3\}$ is given. It is calculated for four different cases: 1) Theoretically, assuming that it a multinomial distribution. The underlying distribution $P_i$ is given by the manual scoring results, $P_1 = 0.015$, $P_2 = 0.975$, and $P_3 = 0.004$, 2) Manual counting, 3) Automated counting, and 4) Automated counting + correction.

<table>
<thead>
<tr>
<th>Chromosome 8</th>
<th>N</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Theoretical $\sigma_i$</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Multinomial</td>
<td>500</td>
<td>0.5%</td>
<td>0.7%</td>
<td>0.3%</td>
</tr>
<tr>
<td><strong>Manual $s_i$</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spectrum Orange™</td>
<td>800</td>
<td>0.8%</td>
<td>0.1%</td>
<td>0.2%</td>
</tr>
<tr>
<td><strong>Automated $s_i$</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Slide # 1-13</td>
<td>425</td>
<td>1.6%</td>
<td>3.4%</td>
<td>1.4%</td>
</tr>
<tr>
<td><strong>Automated + Correction $s_i$</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Slide # 1-13</td>
<td>425</td>
<td>0.8%</td>
<td>1.4%</td>
<td>0.6%</td>
</tr>
</tbody>
</table>

Besides the systematic difference among the three counting methods, the variance is also an important aspect. We have calculated the standard deviation of the dot distribution using the results of 13 slides. For each slide an average of 425 nuclei has been analyzed. Table 5.2 gives the standard deviation of the probability $p_i$ for three bins $i = \{1, 2, 3\}$. Also included is the theoretical standard deviation as defined in eq. 5.2 and the specified standard deviation for manual counting using Spectrum Orange CEP 8. The variance using full automation is significantly larger than with manual counting. If the results are interactively corrected, the variance reduces and approximates the variance of a multinomial distribution.

5.2.2 Error analysis

To get a better insight in the types of errors that occur, the results of 5 slides are evaluated using relocation. Each nucleus with a doubtful automated scoring is relocated under the microscope and examined visually. A nucleus is either counted correctly or one of the following errors is made: split dot, overlapping dots, missed dots, false dots, out of focus, and debris. Table 5.3 gives the error percentage for each type obtained from 2130 nuclei.
Table 5.3: Percentage of cells that are counted incorrectly specified for six different types of error.

<table>
<thead>
<tr>
<th>Error type</th>
<th>N = 2130</th>
</tr>
</thead>
<tbody>
<tr>
<td>Correct</td>
<td>89.3 %</td>
</tr>
<tr>
<td>False dot</td>
<td>1.9 %</td>
</tr>
<tr>
<td>Missed dot</td>
<td>0.8 %</td>
</tr>
<tr>
<td>Split dot</td>
<td>0.4 %</td>
</tr>
<tr>
<td>Overlapping</td>
<td>2.6 %</td>
</tr>
<tr>
<td>Out of focus</td>
<td>2.7 %</td>
</tr>
<tr>
<td>Debris</td>
<td>2.3 %</td>
</tr>
</tbody>
</table>

- Correct — 89 % of all nuclei are counted correctly. In other words, an average of 11 % has to be interactively corrected.
- Split dots — A split dot is counted as two dots instead of one dot. Mis-interpretation of split dots leads to a lower percentage of disomy cells in normal specimen.
- Overlapping dots — Due to segmentation errors, touching or overlapping dots are not properly separated. Two dots are counted as one dot.
- Missed dots — A hybridization dot can be missed during segmentation or is rejected based on its features.
- False dots — Single dots can be separated into two dots or background signal can be detected as a dot. Both errors cause false dots.
- Out of focus — Auto-focusing usually fails because it focused on debris instead of the nuclei. If a nucleus is out of focus the dots will not be detected.
- Debris — Debris, fluorescent material, or air bubbles can not always be distinguished from single nuclei based on the features.

Split dots and false dots yield a false positive rate of 2.3 %. Overlapping, missed dots and out-of-focus errors give a false negative rate of 6.1 %. The results show that the slide quality has great influence on the performance of the automated system. Specimens with less debris and other fluorescent material in the background will reduce the error rates due to fewer false dots, less debris, and fewer focusing errors.

5.2.3 Single monochrome imaging vs. sequential monochrome imaging
The results of table 5.1 have been obtained with single monochrome imaging. To test if the results can be improved with sequential monochrome imaging the performance of single monochrome imaging is compared to sequential monochrome imaging. Table 5.4 gives the automated dot counts of three slides obtained first with single monochrome imaging and then again with sequential monochrome imaging. With both methods the same area of the slide is scanned with the same parameter settings. The results of single monochrome imaging are comparable with the results of sequential monochrome imaging. Independent, sequential color information does not improve the performance of
the dot counter in this case. False positive errors due to the DAPI counter stain are not significant with single monochrome imaging.

Table 5.4: Automated scoring results obtained with single monochrome imaging and sequential monochrome imaging.

<table>
<thead>
<tr>
<th>Chromosome 8</th>
<th>N</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>&gt;3</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Automated</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Slide #4</td>
<td>500</td>
<td>0.4%</td>
<td>2.8%</td>
<td>95.8%</td>
<td>0.8%</td>
<td>0.2%</td>
</tr>
<tr>
<td>Slide #5</td>
<td>501</td>
<td>1.6%</td>
<td>3.4%</td>
<td>92.0%</td>
<td>2.6%</td>
<td>0.2%</td>
</tr>
<tr>
<td>Slide #6</td>
<td>501</td>
<td>6.6%</td>
<td>2.8%</td>
<td>86.4%</td>
<td>3.8%</td>
<td>0.4%</td>
</tr>
<tr>
<td><strong>Automated</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Slide #4</td>
<td>502</td>
<td>0.2%</td>
<td>3.6%</td>
<td>95.4%</td>
<td>0.6%</td>
<td>0.2%</td>
</tr>
<tr>
<td>Slide #5</td>
<td>504</td>
<td>2.0%</td>
<td>4.2%</td>
<td>92.3%</td>
<td>1.4%</td>
<td>0.2%</td>
</tr>
<tr>
<td>Slide #6</td>
<td>500</td>
<td>1.2%</td>
<td>2.6%</td>
<td>92.0%</td>
<td>3.2%</td>
<td>0.4%</td>
</tr>
</tbody>
</table>

5.2.4 Counting results of a trisomy

So far, we have tested the performance of the dot counter with normal specimens because that gave us the opportunity to compare our data with data reported in literature, which was also based on normal specimens. The question remains what the performance will be when tumor specimens are analyzed. To get more insight in the performance of the dot counter in a clinical situation we analyzed two trisomy slides. The slides contained bone marrow and were hybridized with a CEP 8 Spectrum Orange probe. Both samples were from the same patient and have been provided by the Department of Haematology (University of Wales College of Medicine, Cardiff, Wales). Table 5.5 shows the resulting dot distribution of the two slides.

The manual obtained results have been provided by the Department of Haematology. The interactive obtained results are not in agreement with these manual obtained results. This can be explained by a different interpretation of split dots. We were not familiar with their counting procedure. It seems that we have underestimated (manually) the number of trisomy cells because we have counted a number of trisomy cells as two due to a mis-interpretation of split dots. The number of cell that have been counted in-correctly with the automated obtained results was also larger than we expected from the results obtained with normal specimens. Most of the errors are caused by mis-interpretation of split dots and out-of-focus errors. The auto-focusing algorithm failed because it focused on debris or other fluorescent material instead of the nuclei. Most of the nuclei that were not properly focused have been rejected because the dots could not be recognized anymore. This explains the differences between the number of cells that have been used to estimate the dot distribution for the automated obtained results and the results after interactive correction.
Table 5.5: Percentage of cells containing various number of dots for a bone marrow specimen as identified manually, with full automation, and with full automation followed by interactive correction. Slide 1 and 2 were duplicate slides from the same patient. The manual scoring have been provided by the department of Haematology, University of Wales College of Medicine.

<table>
<thead>
<tr>
<th>Chromosome 8</th>
<th>N</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>&gt;3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Manual</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Slide #1 + Slide #2</td>
<td>1000</td>
<td>0.0%</td>
<td>3.8%</td>
<td>50.1%</td>
<td>45.9%</td>
<td>0.2%</td>
</tr>
<tr>
<td>Automated</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Slide #1</td>
<td>590</td>
<td>0.8%</td>
<td>7.1%</td>
<td>55.7%</td>
<td>35.1%</td>
<td>1.2%</td>
</tr>
<tr>
<td>Slide #2</td>
<td>264</td>
<td>2.3%</td>
<td>24.6%</td>
<td>47.3%</td>
<td>23.1%</td>
<td>2.7%</td>
</tr>
<tr>
<td>Automated + Correction</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Slide #1</td>
<td>464</td>
<td>0.0%</td>
<td>4.1%</td>
<td>57.5%</td>
<td>38.1%</td>
<td>0.2%</td>
</tr>
<tr>
<td>Slide #2</td>
<td>236</td>
<td>0.0%</td>
<td>8.9%</td>
<td>59.7%</td>
<td>30.9%</td>
<td>0.4%</td>
</tr>
</tbody>
</table>

One should notice that the results are only based on two slides. Although the error rate with these specimens is higher than with normal specimens we think that the performance can easily be improved. Clean slides will reduce the out-of-focus error rate. Because we had only two slides, it was not possible to do a proper initialization of the parameters and a performance test at the same time. A better initialization of the parameters will improve the results. A more consistent counting procedure will reduce the differences between our results and the results obtained by the Department of Haematology.

5.3 Evaluation of the dot detection algorithms

The performance of the dot detection algorithms has been evaluated. The test set of images has been used with these experiments. The images have been checked manually to exclude focusing errors. The purpose of these experiments was to invest the performance of the different image analysis algorithms.

5.3.1 Counting results

The dot distribution of the test set has been estimated with the three different algorithms: Tophat Threshold, nL Threshold and Dot Label (table 5.6). Together with these fully automated counting results, manually obtained results are also given. Each nucleus from the test set has been counted manually using the monitor display. Again the first row shows the specified dot distribution of chromosome 12 for a normal specimen. The manually obtained results of the test set are comparable to the specifications. Using a monitor display instead of the microscope does not influence the counting result. The automated results differ from the manually obtained dot distribution as we expected. The best result has been obtained with the Dot label algorithm. About 2% of the nuclei are counted incorrectly. Using the Tophat threshold the error percentage increases to 8%.
Table 5.6: Percentage of cells containing various number of dots for the test set as identified manually using a monitor display and with full automation using three different algorithms. Also the dot distribution for normal specimens is given (Vysis). The number of cells that have been counted $N_{cell} = 814$.

<table>
<thead>
<tr>
<th>Chromosome 12</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>&gt;4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Manual</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spectrum Orange™</td>
<td>0.2%</td>
<td>1.5%</td>
<td>97.1%</td>
<td>1.2%</td>
<td>0.1%</td>
</tr>
<tr>
<td>Monitor display</td>
<td>0.6%</td>
<td>1.4%</td>
<td>97.2%</td>
<td>0.6%</td>
<td>0.25%</td>
</tr>
<tr>
<td>Automated</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tophat Threshold</td>
<td>0.6%</td>
<td>8.7%</td>
<td>89.3%</td>
<td>1.1%</td>
<td>0.3%</td>
</tr>
<tr>
<td>nL Threshold</td>
<td>0.5%</td>
<td>3.9%</td>
<td>94.2%</td>
<td>1.0%</td>
<td>0.4%</td>
</tr>
<tr>
<td>Dot Label</td>
<td>0.5%</td>
<td>1.8%</td>
<td>95.1%</td>
<td>2.1%</td>
<td>0.5%</td>
</tr>
</tbody>
</table>

5.3.2 Overlapping dots

The percentage of nuclei containing one dot is significantly larger for the automated results obtained with the Tophat Threshold and nL Threshold than with manual counting. This is especially true when the Tophat Threshold has been used. Most errors occur because "overlapping" dots can not be properly separated. The proportion $\varepsilon_o$ of nuclei that are counted as one instead of two has been obtained.

Table 5.7: Because "overlapping" dots can not always be properly separated some nuclei are counted as having one dot instead of two. The proportions of "overlapping" dots $\varepsilon_o$ as a percentage of the total number of cells containing two dots observed with the three different algorithms are given, together with the measured ratio $r_o/R_c$ and the calculated probability of an overlap. The number of cells with two dots is $N_{o} = 800$.

<table>
<thead>
<tr>
<th>Algorithm</th>
<th>$\varepsilon_o$</th>
<th>$r_o/R_c$</th>
<th>$P_o(r_o/R_c)$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tophat Threshold</td>
<td>7.1±1.8%</td>
<td>0.26±0.02</td>
<td>7.6±1.2%</td>
</tr>
<tr>
<td>nL Threshold</td>
<td>2.4±1.0%</td>
<td>0.17±0.01</td>
<td>3.1±0.4%</td>
</tr>
<tr>
<td>Dot Label</td>
<td>0.9±0.6%</td>
<td>0.13±0.01</td>
<td>1.9±0.3%</td>
</tr>
</tbody>
</table>

The results of the three algorithms are examined visually using the monitor display. Each nucleus that has an automated scoring of one dot has been classified manually as a single dot or an "overlapping" dot. Table 5.7 shows the number of "overlapping" dots for each algorithm as a percentage of the number of nuclei containing two dots. The error interval is defined as two times the standard deviation given by eq. 5.2. The smallest distance $r_o$ that can be separated is estimated using the frequency distribution of the distances between two dots. The distance between two dots is normalized by the radius of the nucleus $R_c$. The result is also given in table 5.7, together with the estimated probability of an overlap $P_o$ (see section 4.3.1).
Figure 5.1: The frequency distribution histogram of the projected distance between two dots within a nucleus. The distance is normalized by the radius of the nucleus. The measured distribution curves are given for all nuclei with two detected dots obtained 1) with the Tophat Threshold and 2) with the distribution of a model which assumes uniform and independent distribution of point-like dots.

For each algorithm the distances between two dots have been measured for all nuclei with two correctly detected dots. Because the frequency distribution of distances does not contain those cells for which the dots are not properly separated, we may expect that the smallest distance that occurs in the distribution is the smallest distance for which the dots can be separated. However, it is possible that the smallest distance of the distribution is an outlier caused by noise. In other words, two touching dots, at a certain distance, are sometimes properly separated and sometimes not. Therefore $r_o$ is defined as the distance at the 0.5% level of the cumulative frequency distribution. In our case with $N \approx 800$, $r_o$ becomes equal to the fourth smallest distance (sample) in the distribution. The error interval is defined as the difference between the third smallest distance and the fifth smallest distance. Figure 5.1 shows the measured frequency distribution as a result of the Tophat Threshold in combination with the distribution of a model which assumes a uniform and independent distribution of point-like dots. The model frequency distribution is obtained from simulations. Figure 5.2 shows the cumulative distributions for the three algorithms with the resulting $r_o$ distance at the 0.5% level. The Dot Label algorithm has the best performance in segmenting "overlapping" dots followed by the nL Threshold. The nL filter significantly improves the result of the Tophat Threshold. There is no
significant difference between the estimated probability of an overlap based on the model and the measured proportion of "overlapping" dots $\varepsilon_o$, but the error intervals are larger.

![Graph](image)

**Figure 5.2:** The cumulative frequency distribution curves of the projected distance between two dots as observed in cells with two detected dots. The distance is normalized by the radius of the nucleus. The curves have been obtained with the three algorithms. The 0.5% level defines $r_o$. Only a small range near zero is plotted.

### 5.3.3 Overlapping dots detection

Including an extra classification step to distinguish "overlapping" dots from single dots has been evaluated. The result of the Tophat Threshold has been used to see if it is possible to improve the results based on the total intensity and eccentricity.

**Table 5.8:** The mean and standard deviation of the total intensity and the eccentricity for single dots and "overlapping" dots. The result of the Tophat Threshold is used to calculate these values.

<table>
<thead>
<tr>
<th>Features</th>
<th>Total Intensity (ADU)</th>
<th>Eccentricity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\mu$</td>
<td>$\sigma$</td>
</tr>
<tr>
<td>Single dots ($N = 1452$)</td>
<td>7458</td>
<td>4079</td>
</tr>
<tr>
<td>Overlapping dots ($N = 57$)</td>
<td>14823</td>
<td>7677</td>
</tr>
</tbody>
</table>

Table 5.8 gives the mean and standard deviation of the total intensity and the eccentricity for "overlapping" dots and single dots. The number of single dots is much larger than the number of "overlapping" dots because we have used all dots from the cells with two detected dots. As we expected, the average total intensity of "overlapping" dots is twice
the total intensity of single dots. Also the eccentricity is larger for "overlapping" dots. The variation, however, is also larger. A scatter plot (figure 5.3) illustrates the overlap between the two classes.

![Scatter plot of eccentricity vs total intensity](image)

**Figure 5.3:** Scatter plot of the total intensity and the eccentricity for single dots (light gray) and "overlapping" dots (dark gray).

A nearest neighbor (NN) classifier (Fukanaga 1990) has been applied to these data. The set of 1452 single dots and 57 "overlapping" dots has been used as a training set and as a test set. Because the number of "overlapping" dots is small, we did not want to split the set into a separate training set and test set. Table 5.9 gives the result of 6-NN classifier.

**Table 5.9:** A confusion matrix for a 6-NN classifier using the total intensity and the eccentricity to distinguish "overlapping" dots from single dots. The learning set was also used as test set. More than 99% of the single dots are classified as single dots but only 54% of the "overlapping" dots are classified correctly.

<table>
<thead>
<tr>
<th>Classification \ Test set</th>
<th>Single dots ($N = 1452$)</th>
<th>Overlapping dots ($N = 57$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Single dots</td>
<td>99.7%</td>
<td>45.6%</td>
</tr>
<tr>
<td>Overlapping dots</td>
<td>0.3%</td>
<td>54.4%</td>
</tr>
</tbody>
</table>

Because the training set has been used as the test set these results are optimistic. The classifier has been trained in such a way that the number of false negatives (single dots that are classified as "overlapping" dots) is close to zero. Only 54% of the "overlapping"
dots are classified correctly. This result shows that an extra classification step only slightly improves the result. The large variation of the features makes it difficult to distinguish "overlapping" dots from single dots.

5.3.4 Split dot detection

The ability to separate touching dots means that a split dot could be detected as two dots. Mis-interpretation of a split dot can lead to a high percentage of trisomy cells in normal specimens. A simple rule has been used to detect split dots based on the distance between the two dots and the relative intensity. This rule is only applied on nuclei with more than two dots. If two dots are classified as a split dot, these dots are combined into one dot.

Table 5.10: The proportion of cells that have been counted incorrectly due to a mis-interpretation of a split dot. The percentages are given for the nL Threshold algorithm and the Dot Label algorithm with and without split dot detection. The split dot detection combines most of the split dots.

<table>
<thead>
<tr>
<th>Algorithm</th>
<th>No split dot detection</th>
<th>Split dot detection</th>
</tr>
</thead>
<tbody>
<tr>
<td>nL Threshold</td>
<td>3.2%</td>
<td>0.4%</td>
</tr>
<tr>
<td>Dot Label</td>
<td>9.1%</td>
<td>1.2%</td>
</tr>
</tbody>
</table>

To test this rule the test set has been analyzed again using the three algorithms but without split dot detection. The results given in table 5.6 were obtained with the use of split dot detection. The results of the two experiments are now compared. Table 5.10 gives the proportion of nuclei that have been counted incorrectly because of a split dot, with and without the use of split dot detection. Because the Tophat Threshold does not have the ability to separate touching dots, no split dots have been detected as two dots and the Tophat Threshold is therefore not included. If the ability to separate dots improves, the number of split dots that have been detected as two dots increases. Using the split dot detection, most of the split dots have been combined into one. Four nuclei have been counted incorrectly due to split dots using the Dot Label algorithm and cause a slightly higher percentage of trisomies (table 5.6).

5.4 Non-flat nuclei

The error analysis in section 5.2.2 showed that a dot can be missed when it is out-of-focus. With those experiments the auto-focusing algorithm failed because it focused on debris instead of the nuclei. Visual examination of the slides showed that when a FOV is in-focus all the dots are in-focus. This means that the nuclei are flat and that they are all in the same focal plane. This will not always be the case. Non-flat nuclei will influence the scoring result of the dot counter. To measure the influence on the performance we have tested the system with other slides. With these slides it was not possible to focus all
the dots within one FOV at the same z-position. The slides also contained lymphocytes from cultured blood and were hybridized with a Spectrum Orange CEP 8 probe. Due to a different preparation technique the nuclei were not as flat as with the other slides.

![Graph showing focus functions of four dots within a single image of a non-flat slide.](image)

**Figure 5.4:** The focus functions of four dots within one image of a so called "non-flat" slide. The focus position varies from 1.75 μm to 6.25 μm.

### 5.4.1 Focus position of the dots

Before the counting results are discussed we want to quantify the flatness of the nuclei. Therefore we have measured the focus position of the individual dots within one FOV and compared the results of a "non-flat" slide with the results of a "flat" slide. A sequence of images has been acquired at different focus positions of two nuclei. A focus step of 0.25 μm has been used. The focus value of a region of 20x20 pixels around the dots has been calculated as a function of the z position. Each dot will produce its own focus function. The position of the maximum of the focus function is the in-focus position of the dot. Figure 5.4 shows the focus function of four dots from a "non-flat" slide. It shows clearly that the differences between the in-focus positions of the dots can be larger than 4 μm. Figure 5.5 shows the result obtained with a "flat" slide. In this case the focus functions are more or less at the same z position. We could now define a measure of the flatness of a slide as the average standard deviation of the focus positions of the dots per FOV. We have measured the standard deviation of the in-focus positions of 4 dots within one image for 5 FOV's. The average standard deviation of the "non-flat" slide and the "flat" slide are 1.2 μm and 0.16 μm, respectively.
Figure 5.5: The focus functions of four dots within one image of a so called "flat" slide. All the dots are in the same focal plane.

5.4.2 Counting results
The dot distribution of the "non-flat" slide has been obtained with the automated dot counter. Table 5.11 gives the results of the dot counter with and without interactive correction by the user. Because the x40/1.3 objective has a small depth-of-focus we have also used a x25/0.8 objective to increase the depth-of-focus. Considering the automatically obtained results the dot distribution shows a much higher percentage monosomy cells compared to the results given in table 5.1.

Table 5.11: Percentage of cells containing various number of dots for a "non-flat" slide as identified manually using a monitor display and with full automation using the Dot Label algorithm. Two different objectives have been used. The number of cells that have been counted $N = 540$. Because not all the dots are in-focus the automatically as well as manually obtained dot distribution differs from the expected dot distribution of a normal specimen.

<table>
<thead>
<tr>
<th>Chromosome 8</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>&gt;4</th>
</tr>
</thead>
<tbody>
<tr>
<td>40x/1.3 objective</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dot Label algorithm</td>
<td>0.9%</td>
<td>31.0%</td>
<td>63.9%</td>
<td>4.0%</td>
<td>0.0%</td>
</tr>
<tr>
<td>Monitor display</td>
<td>0.7%</td>
<td>7.0%</td>
<td>90.7%</td>
<td>1.4%</td>
<td>0.2%</td>
</tr>
<tr>
<td>25x/0.8 objective</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dot Label algorithm</td>
<td>1.6%</td>
<td>11.0%</td>
<td>79.2%</td>
<td>6.9%</td>
<td>1.5%</td>
</tr>
<tr>
<td>Monitor display</td>
<td>0.4%</td>
<td>2.9%</td>
<td>92.5%</td>
<td>2.6%</td>
<td>1.7%</td>
</tr>
</tbody>
</table>
Because not all the dots are in focus they have not been detected properly. It is interesting to see that the manual scoring also differs from the expected dot distribution. If the focusing error becomes too large a dot cannot be recognized on a monitor display. Using a low NA objective improves the results but the error rate is still much larger when compared to the error rate of 11% given in section 5.2.2. The number of trisomy cells is also higher than we expected. Because the measurement of the intensity features is influenced by the focus position, split dots are not always detected.

5.5 Screening time

An analysis of screening time for the complete system is given in table 5.12. The screening time is calculated for low cell density slides (1 cell/image) as well as for high density slides (10 cells/image). The total screening time depends strongly on the cell density. Scanning a high density slide instead of low density slide reduces the screening time by more than a factor of 6!

<table>
<thead>
<tr>
<th>Action per Image (sec.)</th>
<th>Cells per Image</th>
<th>Low Density</th>
<th>High Density</th>
</tr>
</thead>
<tbody>
<tr>
<td>Move Stage</td>
<td></td>
<td>0.4</td>
<td>0.4</td>
</tr>
<tr>
<td>Focus</td>
<td></td>
<td>9.6</td>
<td>9.6</td>
</tr>
<tr>
<td>Integrate</td>
<td></td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Transfer</td>
<td></td>
<td>1.8</td>
<td>1.8</td>
</tr>
<tr>
<td>Find ROI's</td>
<td></td>
<td>0.6</td>
<td>0.6</td>
</tr>
<tr>
<td>Analyze ROI</td>
<td></td>
<td>0.7</td>
<td>7.0</td>
</tr>
<tr>
<td>Combined (parallel) Ops.</td>
<td></td>
<td>(1.4)</td>
<td>(1.4)</td>
</tr>
<tr>
<td>Time / image (sec.)</td>
<td></td>
<td>12.7</td>
<td>19.0</td>
</tr>
<tr>
<td>Time / cell (sec.)</td>
<td></td>
<td>12.7</td>
<td>1.9</td>
</tr>
<tr>
<td>Time / 500 cells (min.)</td>
<td></td>
<td>106</td>
<td>16</td>
</tr>
</tbody>
</table>

The time that is saved by parallel operations is 1.4 sec. and consists of the integration time (1.0 s) and the time for moving the stage (0.4 s). Because the image analysis time is longer then the integration time, a faster computer will speedup the system. The image analysis will be completed before the integration of the next image is finished if an 8x (processing time for high density slides / integration time, 7.8/1.0 = 8) faster computer is used. The time to analyze 500 nuclei will then be reduced to 10 min. With such a computer the total screening time will be mainly limited by the integration time and the readout time of the camera. To reduce the screening time even more, a camera with a
faster readout should be used or the brightness of the specimen must be increased (or both). For example using the Xillix Micro Imager, the time to analyze 500 nuclei will be about 5 min. This camera uses the same CCD chip as the Photometrics camera but has a 16x faster readout rate.

5.6 Discussion

We have developed a completely automated fluorescence microscope system that can examine interphase cell nuclei in order to determine the proportions of cells containing 0, 1, 2, 3, or >3 dots. The fully automated enumeration of chromosomes in doubly stained cells is possible.

5.6.1 Overall performance
The performance has been tested in a number of experiments with normal specimens. The results of this system are compared with manual counting. The automatically determined dot distribution is biased with respect to manual counting. Also the variance is higher than with manual counting. An average of 11% of the nuclei is counted incorrectly. Error analysis showed that the errors can be divided into six different error types: false dots, missed dots, split dots, overlapping dots, out of focus, and debris.

The slide quality has a significant influence on the performance. Debris, high auto-fluorescence and low probe intensity can make results unreliable. Focusing errors due to non-flat nuclei cause a much higher number of monosomy cells. After correction the accuracy is generally comparable to manual counting with the exception of focusing errors. If a dot is too far out-of-focus it cannot be recognized on a monitor display.

The comparison between single monochrome imaging and sequential monochrome imaging shows that separated color channels do not improve the performance of the dot counter. This result depends on slide preparation and will not be the case in general. For example if the intensity of the counter stain is too bright, the contrast with the dots will be low and sequential monochrome imaging is preferable. The advantage of single monochrome imaging is that the scanning speed is faster than with sequential monochrome imaging. But single monochrome imaging is only applicable to slides with a single hybridization. It is not possible to distinguish two different probes labeled with two different dyes plus a counter stain, based upon their gray level intensities. To automatically analyze multiple hybridized specimens, sequential monochrome imaging must be used.
5.6.2 Dot detection algorithms

The results of the Dot Label algorithm approximates the manually obtained results. Only 2% of the cells are counted incorrectly. Using nL Threshold or Tophat Threshold the error rates increase to, respectively, 3% and 8%. Because the images of the test set have been checked manually the error rate of the complete system, automated scanning and image analysis, will be higher. The error analysis of the complete system showed an error rate of 3% (out of the total 11%) to be caused by focusing errors.

"Overlapping" dots are the main problem of dot segmentation. The overall error rate is strongly related to the ability to separate touching dots. The estimated distance \( r_o \) gives an indication of how well the dots can be separated. The smallest distance has been measured with the Dot Label algorithm. The estimated error percentage due to an overlap is comparable to the calculated probability of an overlap using the distance \( r_o \) (see section 4.3.1). Of course this result depends on how we have defined \( r_o \). The distance \( r_o \) has been defined as a fixed distance. In other words two dots at a distance smaller than \( r_o \) will not be separated, and if the distance is larger they will be separated. The frequency distribution of the distances between two dots, as a result of the Tophat Threshold in comparison with the results of the model, shows that it is not a fixed distance but an interval where some dots are separated and some are not. If the distance between the dots becomes larger, the distribution of the model, which assumes uniform and independent distribution of point-like dots, is in agreement with the result of the Tophat Threshold. The underlying assumption of the calculated probability of an overlap seems to be reasonable. Although the definition of \( r_o \) is ambiguous, the calculated probability yields a good approximation for the measured probability of an overlap.

Including an extra step, that distinguishes "overlapping" dots from single dots based on the eccentricity and total intensity, has been tested. Although the total intensity of "overlapping" dots is twice the total intensity of a single dot, and the eccentricity is larger for "overlapping" dots, the large variation of the features yields only a small improvement of the results.

If the ability to separate touching dots increases, the number of split dots that are detected as two dots will increase. A simple rule is applied to detect split dots. If two dots are classified as a split dot these two dots are combined. Without the split dot detection, the results of nL Threshold and Dot Label would have a higher estimated percentage of trisomy cells and the results would be unreliable. The split dot detection combines most of the split dots. We have only tested the split dot detection on a normal specimen. Therefore we could not measure the false negative rate of the split dot detection. The false negative rate has been defined as the percentage of real trisomy cells that are counted as two dots because of a false detection of a split dot. None of the few trisomy
cells in the test set has been detected as a split dot. The number of trisomy cells is not sufficient to give an estimation of the false negative rate.
Chapter 6

General Discussions
Developments in digital imaging technology have made it possible to use integrated microscope systems for the analysis of FISH preparations. FISH methodology in combination with digital imaging techniques have become an important tool to identify and quantify structural and numerical chromosome aberrations. Multiple target can be visualized with fluorescent labels. The major disadvantage associated with the use of fluorescent dyes is the weak signal emanating from a probe. These signals cannot be detected with a conventional camera at video rate. The weak intensities require a camera that can integrate for several seconds, and a proper setup of the microscope system. A completely automated microscope system consists of a fluorescence microscope, motor driven stage, filter and focus control, an imaging device, and a computer to control the whole process. All these components are commercially available today. The system described in this thesis is built around an ordinary fluorescence microscope and is controlled by a personal computer. No special hardware has been used. A critical part of the system is the imaging device. The characterization of the Sony camera showed that there are some drawbacks when a normal video based camera is used in fluorescence microscopy. The low efficiency in the UV range and near the infra-red is not desirable. The efficiency can be improved by a special coating that improves the UV response. The IR filter, which is not necessary in fluorescence microscopy, could be removed. In that case such a video camera would offer a reasonable performance for a low price. The Photometrics has an excellent performance but is very expensive. In general FISH applications do not require such a high performance CCD camera. Less expensive cameras are available with a comparable performance.

We have focused on one particular application, chromosome enumeration. The system contains all components common to image processing and image analysis: auto-focusing, segmentation, measurements, and classification. We have developed a system that counts fluorescent hybridization dots for one probe in interphase cell nuclei. We have shown that automated enumeration of chromosomes in single hybridized interphase cells is possible. The system can easily be extended to a system that can analyze multiple hybridized preparations. The different probe should be labeled with different dyes and each dye must be acquired in a separated channel using sequential monochrome imaging.

The performance of the dot counter has been tested in a number of experiments with normal specimens. The automatically determined dot distribution is biased with respect to manual counting. Also the variance is higher than with manual counting. Using the nL Threshold algorithm, an average of 11% of the nuclei are counted incorrectly. The Dot Label algorithm will slightly reduce the error rate due to the improved ability to separate touching dots. Split dots and "overlapping" dots are the main problems of the dot detection algorithm. The error percentage caused by false dots and missed dots is strongly related to the slide quality and focusing errors. If the images are from a clean slide (no
General Discussions

debris and no minor bindings) and the dots are in-focus, the false positive and false negative error rates are significantly reduced for all three algorithms. In that case the Dot Label algorithm approximates the accuracy of manual counting. The image analysis is done at a reasonable speed and accuracy with common image processing tools on a personal computer.

Scanning time analysis showed that the speed of automated dot counting is currently comparable to human counting (without taking human fatigue into account). The speed can easily be increased by another factor of 3. The improvements in the next generation system will be based upon a faster computer (8x) and a faster camera transfer (16x). Using TDI scanning instead of start-stop scanning will increase the speed even more. The speed-up depends on the integration time and the readout time of the camera. For an integration time $t_i = 1.0$ sec. the speed-up will be 1.3 and 1.16 for a readout rate of 500 kHz and 8 MHz, respectively. The auto-focusing time has not been taken into account. Compared to start-stop systems, TDI scanning offers a speed improvement, negligible blurring in the scanning direction and a complete suppression of pixel variability boosting the image SNR more than 10 dB.

A user-friendly interface is an important part of the system because the human eye is still the gold standard. A number of tools are provided to control the microscope and evaluate the results. It is possible to relocate nuclei under the microscope, select nuclei based on their features, and interactively correct the dot counts. The latter is essential for such a system because the automatically obtained results are not perfect. Results showed that after interactive correction the accuracy is comparable to manual counting. The user can review and correct the data in a rapid manner through the use of galleries. To review 500 nuclei we need less than 5 min. behind a computer monitor. The microscope has not been used and that has the advantage that we do not have to work in a dark environment. Such an approach will be of help for analyzing large quantities of slides and large number of cells, as manual scoring is tedious and time consuming.

The slide quality has a significant influence on the performance. Both the accuracy and speed depend on the preparation. Debris, high auto-fluorescence and low probe intensity can make results unreliable. The requirements of a specimen for automated dot counting may differ from those for manual counting. The following characteristics are desirable:

- Fixed region — The cells should be at a fixed region on the slide. If not, an operator has to define a scan area for each slide. Although it is relatively easy to find that region manually, it is difficult and time consuming to automate this task. A fixed region on a slide is a simple solution.
• High cell density — The scan speed is strongly related to the cell density. A cell density of more than 10 cells/image is necessary to achieve a reasonable scan time of 15 min. in order to analyze 500 nuclei. A higher cell density will reduce the scan time even more until the cell density becomes so high that a non-negligible fraction of nuclei overlap and segmentation performance deteriorates.

• Homogeneous cell distribution — Cells on a slide have the tendency to form clusters. Cell clusters are difficult to analyze and the analysis is time consuming. On the other hand focusing will fail on empty fields. The latter can be prevented by first checking if there are cells in the image.

• Flat nuclei — Because high NA objective has been used the depth-of-focus is small. Results showed that dots can be missed when they are not in the same focal plane. A low NA objective increases the depth-of-focus but reduces the brightness and therefore increases the scan time. But even a low NA objective is not always sufficient to get reliable results. A three-dimensional image analysis approach should be applied. Instead of acquiring one image per FOV, a number of images have to be acquired at different focus positions. The two dimensional image processing algorithms have to be modified. One can use the two-dimensional image processing algorithm for each plane and combine the binary output images to detect the dots, or the two-dimensional algorithm has to be extended to a three-dimensional algorithm. About 5 images per FOV should be sufficient to cover a focus range of 10 µm which is on the order of a cell diameter. The focus position of a dot is then always within 1 µm of one the five planes. In that case the reduction of the maximum intensity of a dot will be smaller than 10% (see chapter 4). The disadvantage of this approach is that the whole procedure will be at least 5 times slower.

• Clean slides — Debris, fluorescent material and background fluorescence can make the results unreliable. Sometimes it is not possible to distinguish fluorescent material from real single cell nuclei based on their features. The auto-focusing algorithm can fail if it focuses on some fluorescent material that is not in the same focal plane as the nuclei. Besides a reduction of the number of cells that are counted incorrectly, clean slides will also reduce the variance of the fully-automated obtained results. Our experience is that the large variation between the results of different slides is always related to the slide quality. Clean slides will make the error rate of the dot counter more predictable. If the false positive and false negative error rates are known and stable, it is possible to estimate the real dot distribution from the automatically obtained results and the interactive correction of the results will not be necessary anymore.
Finally, the performance of the dot counter has been tested mainly with normal specimens. The limited number of trisomy slides does not make it possible to draw definite conclusions from these results. To get more insight in how robust the automated dot counter is and how the performance is related to different preparation techniques the system should be evaluated in a clinical environment.

Considering the future of automated dot counting there is an important difference between being used in a clinical environment or as a research tool. In a clinical environment a dot counting system will be used for routine cytogenetic tests and the system will be more or less a black box. Slides will be automatically loaded into the system and after the analysis results can be evaluated and interpreted. Because such a system makes use of complex instrumentation, automated calibration and error check procedures must be included. Because the slide quality (preparation and FISH staining) has great influence on the performance of the dot counter, the slide preparation should be optimized for automation. Besides the aspects mentioned above like high cell density, flat nuclei, and homogeneous distribution, the quality of the FISH staining should be constant. The accuracy of the system will be more predictable and the user will have more confidence in the results. In the ideal case the slide preparation would be automated too. All conditions could be controlled and the variation would be reduced to a minimum. Automation of this process, if possible, will be complicated and at the end there will always be some biological variation.

The choice between manual counting and automated counting depends on the number of slides that have to be scanned, the number of cells per slide that have to be analyzed, and the complexity of the analysis. In the simple case of a single hybridization and only a few hundred cells that have to be analyzed (a low required accuracy in combination with a high proportion of aberrant cells), it is difficult to be competitive with manual counting. Only when the number of slides is large, can automation be cost effective in this situation. However automation would provide many advantages in case where it is desirable to screen large numbers of cells (10,000 or more) for evidence of malignant cells present at low frequency and subsequently to accurately monitor changes in the frequency of these cells (e.g. patient monitoring). If the complexity of the analysis increases, for example multiple hybridization or ratio labeling, manual counting would be more difficult and therefore time consuming; automation could be a solution. Finally automated dot counting has great potential in the analysis of tumor tissue sections. Manual counting in tissue sections is complicated. The difficulties with such a system are not only related to dot detection but also to the cell segmentation within clusters. This would require a 3D approach in which correction procedures, for the truncation effect that occurs in 5 μm sections, are applied.

In a research environment the characteristics of such a system will be different. It will not be used on a routine basis for a limited number of tests, but more as research tool to
analyze a limited number of slides various types of specimens, hybridized with different probes and labeled with a variety of dyes. Because in most of the experiments only a small number of slides are involved, the preparation technique will not always be optimized for automation. In that case the accuracy can not be guaranteed and more user-interaction will be required. The diversity of the samples requires that a system be flexible. The system must be able to work with different filter sets, different objectives and easy to set-up for a specific configuration. The user-interface will be an essential part of the system. The present prototype system provides the user with a number of tools to control the microscope, to adjust parameters, and to evaluate and to correct the results. The user can review and correct the data in a rapid manner through the use of galleries. Future experience with the system will give more insight into which tools are desirable and which one are not practical. Compared with manual counting, the automated dot counter has the advantage that most of the work can be done behind a computer monitor and results will immediately be displayed in a convenient way. Besides the actual dot count the system can provide the user with other quantitative information about the cells and dots that can not be obtained manually.
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Summary

Automated Image Analysis of FISH-Stained Cell Nuclei

In this thesis we describe the development of an automated system for the analysis of FISH specimens. The system contains all of the components common to image processing and image analysis: automated focusing, image acquisition, segmentation, measurement, and classification. The instrumentation that is necessary for the analysis of FISH preparations is the main topic of this thesis. Fluorescence \textit{in situ} hybridization (FISH) is a method to detect specific DNA sequence in interphase cell nuclei as well as metaphase spreads. FISH signals in interphase cells become visible as colored dots. We have focused on one particular application, chromosome enumeration. Chromosome enumeration is commonly called dot counting. With chromosome enumeration, dots are counted for a large number of cells to determine the distribution of chromosomes per cell and to be able to detect small aberrant sub-populations.

\textbf{Chapter 1} provides some background information on fluorescence \textit{in situ} hybridization and the relation with digital image analysis. Biological aspects, such as DNA, chromosomes, mutations and the cell cycle are briefly discussed. Modern molecular technology has made it possible to selectively stain various DNA sequences on biological cells. These sequences may be chosen so as to detect specific abnormalities or to facilitate the process of counting chromosome types. The visualization is achieved with labels based on fluorescence. Using a fluorescence microscope the fluorescent dyes become visible as colored regions. This technique is called fluorescence \textit{in situ} hybridization. FISH techniques have great potential both in research and in clinical applications, for example prenatal diagnosis, early relapse detection in leukemia's and tumor analysis.

Digital imaging technology plays an important role in the development of FISH applications. Modern camera devices allow the detection and visualization of fluorescence signals that can not be detected by the human eye. Quantitative data can be obtained and certain tasks can be automated using image processing and image analysis techniques.
Chapter 2 describes the whole image acquisition procedure. The image acquisition procedure covers the process that starts with a certain specimen and results in the digitized images stored in computer memory. A complete system consists of an automated fluorescence microscope, a camera to digitize the images, and a computer that controls the entire process. Scanning a specimen consists of a number of steps that are repeated until a preset number of cells is analyzed. Each field-of-view is focused automatically after which an image is acquired. When the acquisition is finished, the image is processed and the microscope stage is moved to the next field-of-view. The goal of this chapter is to get a better insight into the performance of the image acquisition system and how the different parameters, like magnification, numerical aperture and integration time, can be chosen based on the requirements of an application.

Chapter 3 describes the development of a special imaging system for high speed image acquisition in fluorescence microscopy. The use of a two-dimensional CCD array in a special operation mode called TDI (Time Delayed Integration) permits a significant increase in photon integration time compared to 1D scanners (higher signal-to-noise ratio) without compromising the total data throughput rate. Instead of a start-stop system we use continuous stage motion in the CCD's parallel shift direction. Synchronizing the parallel clock and the stage velocity guarantees a one-to-one relationship between a moving cell and its image onto the CCD. Compared to start-stop systems, TDI scanning offers a speed improvement, negligible blurring in the scanning direction and a complete suppression of pixel variability boosting the SNR more than 10 dB.

Chapter 4 describes the image processing algorithms that have been developed for the automated dot counter. Dot counting involves two segmentation steps, the detection of the nuclei followed by the detection of the individual dots. The basic concept of both segmentation procedures is straightforward. The original image is first processed to define a mask for each nucleus. A number of features of each object are measured. The features are used to select single nuclei. Nuclei that are not properly segmented, touching nuclei and debris are rejected. To detect the dots the image is segmented again within the mask of the nucleus. Features of a detected dot are measured. These features can be used to verify if a detected dot is a real single hybridization dot, to detect split dots and to detect overlapping dots. After the dots are detected it is easy to count the number of dots and update the results. A user-interface is included to interactively verify and correct the scoring.

The performance of the automated dot counter has been evaluated and results are given in chapter 5. The system has been tested on a number of normal specimens where a centromeric probe was used to mark the desired chromosome. The slides contained lymphocytes from cultured blood. We have compared the results of the dot counter with
manual counting. Manual obtained results, published in literature, has been used as the "ground truth". The machine accuracies, after interactive correction, are comparable to panels of human experts (manual). The fully automatically obtained results are biased with respect to manual counting. An error analysis is given and different causes are discussed. The slide quality has a significant influence on the performance. Both the accuracy and speed depend on the preparation. Debris, high auto-fluorescence and low probe intensity can make results unreliable. Scanning time analysis showed that the speed of automated dot counting is currently comparable to human counting. The speed can easily be increased by another factor of 3.

Chapter 6 provides a general discussion on various aspects of automated dot counting. Automated chromosome enumeration is possible. Beside the actual dot count the system can provide the user with all kind of quantitative information of the cells and dots, which can not be obtained manually. A user-friendly interface is an important part of the system because the human eye is still the gold standard. A number of tools are provided to control the microscope and evaluate the results. The user can review and correct the data in a rapid manner through the use of galleries. To review 500 nuclei we need less than 5 min. behind a computer monitor. The microscope has not been used and that has the advantage that we do not have to work in a dark environment. Such an approach will be of help for analyzing large quantities of slides and large number of cells, as manual scoring is tedious and time consuming.
Samenvatting

Geautomatiseerde beeldanalyse van FISH-gekleurde celkernen

Dit proefschrift beschrijft de ontwikkeling van een geautomatiseerd systeem voor de analyse van FISH preparaten. Het systeem bestaat uit diverse componenten die binnen het vakgebied van de beeldverwerking en beeldanalyse horen: automatisch focussen, beeld opname, segmentatie, meten en classificeren. Ook de instrumentatie die nodig is voor de analyse van FISH preparaten is het onderwerp van dit proefschrift. Fluorescentie in situ hybridisatie (FISH) is een methode om specifieke DNA sequenties te detecteren in zowel interfase als ook metafase kernen. De FISH signalen in interfase kernen worden zichtbaar als gekleurde spots. We hebben ons gericht op een toepassing; het tellen van chromosomen in interfase kernen. Dit wordt ook wel het tellen van spots genoemd. Bij het tellen van chromosomen worden de spots in een groot aantal cellen geteld zodat de onderliggende verdeling van het aantal chromosomen per cel kan worden bepaald en het mogelijk is kleine afwijkingen te detecteren.

Hoofdstuk 1 vormt een algemene inleiding over fluorescentie in situ hybridisatie en de relatie met digitale beeldverwerking. Begrippen als DNA, chromosomen, mutaties en cel deling worden kort besproken. FISH maakt het mogelijk om selectief DNA sequenties te kleuren in biologische cellen. Deze sequenties kunnen zo worden gekozen dat hiermee specifieke afwijkingen kunnen worden gedetecteerd. Ook is het mogelijk om het aantal chromosomen van een type in een celkern te bepalen. Voor de visualisatie van deze FISH-probes wordt gebruik gemaakt van labels bestaande uit fluorescentie kleuring. Met behulp van een fluorescentie microscoop worden de labels zichtbaar als gekleurde gebieden. Mogelijke toepassingen van FISH liggen zowel in het biomedisch onderzoek als in een aantal klinische toepassingen zoals voor prenatale diagnostiek, detectie van leukemieën en analyse van tumoren.

Digitale beeldverwerking speelt een belangrijke rol binnen de ontwikkeling van FISH toepassingen. Moderne cameratechnologie maakt het mogelijk fluorescentielicht, dat niet met het oog zichtbaar is, te detecteren en te visualiseren. Kwantitatieve gegevens kunnen
worden gemeten en bepaalde taken kunnen worden geautomatiseerd door gebruik te maken van beeldverwerkingstechnieken.

**Hoofdstuk 2** beschrijft de hele beeldopname procedure. Dit is het proces dat begint met een preparaat en resulteert in digitale beelden, opgeslagen in het computer geheugen. Een compleet systeem bestaat uit een geautomatiseerde fluorescentie microscoop, een camera om de beelden te digitaliseren, en een computer die het hele proces aanstuurt. Het scanen van een preparaat bevat een aantal stappen die worden herhaald totdat er genoeg cellen zijn geanalyseerd. Elk microscoopveld wordt eerst automatisch gefocuseerd waarna er een beeld kan worden opgenomen. Na de acquisitie wordt het beeld geanalyseerd en de microscooptafel verplaatst naar het volgende veld. Het doel van dit hoofdstuk is een beter inzicht te krijgen in de prestaties van het acquisitie systeem en hoe de verschillende parameters, zoals vergroting, numerieke apertuur, en integratietijd, kunnen worden gekozen zodat het systeem voldoet aan de eisen van een toepassing.

**Hoofdstuk 3** beschrijft de ontwikkeling van een speciaal beeld acquisitie systeem om met hoge snelheid beelden op te nemen in fluorescentie microscopie. Door gebruik te maken van een 2D CCD array in combinatie met TDI (time delayed integration) is het mogelijk om langer te integreren in vergelijking met een 1D scanner zonder dat de scan snelheid wordt verlaagd. In plaats van een start-stop systeem maken we gebruik van een continue beweging van de microscooptafel in de parallele uitleesrichting van de CCD. Synchronisatie van de parallele uitleesklok en de tafel snelheid garandeert een een-op-een relatie tussen de bewegende cellen en hun beeld op de CCD. In vergelijking tot een start-stop systeem heeft TDI scanen een hogere scan snelheid, een verwaarloosbare bewegingsscherpte, en een volledige onderdrukking van de pixel variatie resulterend in een 10 dB hogere signaal ruis verhouding.

bevat een user-interface waarmee na afloop het resultaat kan worden geëvalueerd en gecorrigeerd.

De prestaties van de automatische spot teller zijn geëvalueerd en de resultaten worden in hoofdstuk 5 beschreven. Het systeem is getest met een aantal normale preparaten (lymfoeyten) waar een centromeer probe is gebruikt om het gewenste chromosoom te markeren. De manueel verkregen spot distributie voor normale preparaten, gepubliceerd in de literatuur, is gebruikt als de gouden standaard voor de onderliggende distributie. De nauwkeurigheid van het systeem is na interactieve correctie vergelijkbaar met manueel tellen. Als er niet wordt gecorrigeerd is er een klein verschil met de verwachte distributie. Er wordt een fout analyse gegeven en verschillende oorzaken worden besproken. De kwaliteit van de preparaten heeft een significante invloed op het resultaat. Zowel de nauwkeurigheid als de scan snelheid is afhankelijk van het preparaat. Debris, hoge autofluorescentie en een lage spot intensiteit kunnen de resultaten onbetrouwbaar maken. Analyse van de benodigde scan tijd laat zien dat de snelheid van het automatische systeem vergelijkbaar is met handmatig tellen. Deze snelheid kan echter makkelijk worden verhoogd met een factor 3.

Hoofdstuk 6 geeft een algemene discussie over verschillende aspecten van de spot teller. Het automatisch tellen van chromosomen in interfase cellen is mogelijk. Naast het aantal spots, kan het systeem ook andere kwantitatieve informatie geven over de cellen en spots, die niet handmatig kan worden verkregen. Een gebruikersvriendelijke user-interface is een essentieel onderdeel van het systeem omdat het menselijk oog uiteindelijk nog steeds de standaard is. Gebruikers functies voor bijvoorbeeld de besturing van de microscoop en evaluatie en correctie van de resultaten zijn eveneens onderdeel van het systeem. De gebruiker kan de data op een snelle manier corrigeren door gebruik te maken van galerijen met beelden van geanalyseerde cellen. Om het resultaat van 500 cellen te corrigeren zijn minder dan 5 min. voor een computer scherm nodig. De microscoop is niet nodig voor de correctie met als voordeel dat men niet in het donker hoeft te werken. Bovendien is automatisch tellen in combinatie met interactieve correctie van groot voordeel bij het analyseren van grote hoeveelheden preparaten en grote aantallen cellen, omdat handmatig tellen tijdrovend en saai is.
Curriculum Vitae

Hans Netten was born in Son en Breugel, The Netherlands on September 30th, 1965. He obtained his VWO diploma from the Scholengemeenschap Bonaventura in Leiden. In 1984 he went to Delft to study Applied Physics at the Delft University of Technology from where he obtained his Master's degree (Ingenieur's diploma) in 1990. The subject of his master thesis was exact reconstruction of sampled images. The research was conducted at the Pattern Recognition Group and supervised by Prof.dr. Ian T. Young.

In 1990 he joined the Pattern Recognition group as a postgraduate researcher and was involved with the development of a fast scanner for fluorescence microscopy. After 18 months he started as a Ph.D. student again at the Pattern Recognition group and continued his work on problems from Quantitative Microscopy. He was involved with development of an automated microscope system for the detection of numerical chromosomal aberrations in interphase nuclei. This project was in collaboration with Vysis (Downers Grove, IL, USA) and the Department of Cytochemistry and Cytometry, University of Leiden.