

A Solid-State Camera System for Fluorescence Lifetime Microscopy

Proefschrift

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CHAPTER 1

Introduction

Abstract

This thesis concerns the measurements of fluorescence lifetime, the techniques which are currently used to measure it, and a new technology we have introduced to improve fluorescence lifetime measurement microscopy (FLIM). Therefore it is important to understand what fluorescence lifetime is and why we want to measure it. This chapter will address these issues and offer an overview about the objectives in this thesis. An outline of the contents of the thesis will be given at the end of this chapter.

Keywords: fluorescence lifetime, fluorescence lifetime imaging microscopy (FLIM)

1.1 Fluorescence and fluorescence lifetime

Fluorescence is a process of photon emission that may occur when a substance absorbs light. When a photon with sufficient energy excites a fluorescent molecule, an electron of the molecule is excited from the ground energy state (S_0) to a higher energy state (S_1 or S_2). These higher energy states have multiple vibrational energy levels, in which the electron can linger for a short period of time. The electrons, however, will quickly relax to the lowest vibrational level of the first energy state (S_1), a process which is called “internal conversion”. The timescale of the internal conversion is 10^{-14} to 10^{-11} seconds [1]. After the vibrational relaxation, the electron drops back to the ground state and emits a photon. This phenomenon can be described in a Jablonski energy diagram, as shown in Fig. 1.1 [2]. The decay from S_1 can occur both by a radiative process (fluorescence emission) as well as by a number of non-radiative pathways (solvent relaxation, intersystem crossing, thermal relaxation, etc.) and a number of excited state reactions (electron transfer, photochromism, photo degradation etc.).

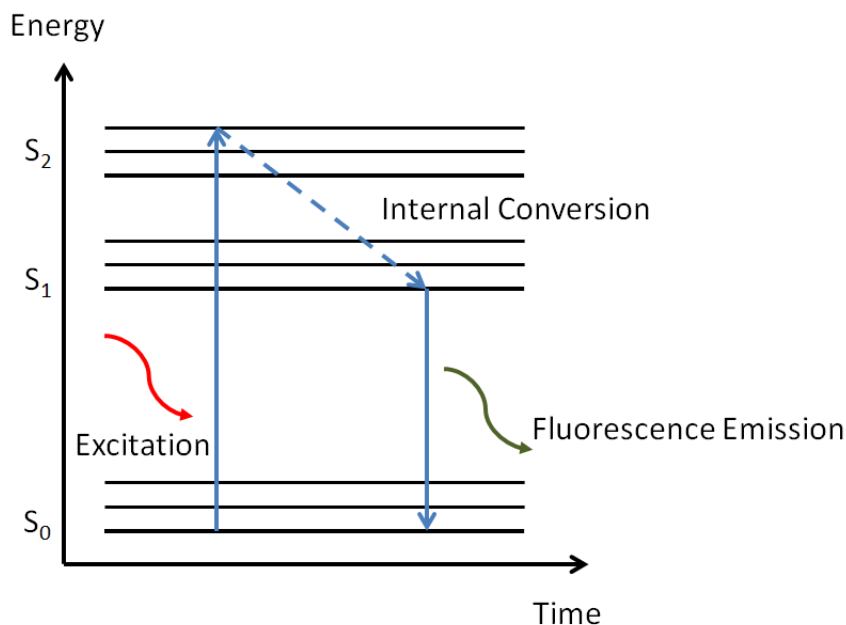


Figure 1.1: Jablonski energy diagram depicting fluorescence.

The emission light will have less energy compared to the excitation light, thus the wavelength of the emission light will be longer than the excitation light. The Stokes shift is defined in this case as the wavelength difference between the maximum of the emission spectrum and the maximum of the excitation spectrum. This Stokes shift of the wavelength makes it possible to design filters to distinguish between emission photons and excitation photons. This will be discussed in the next chapter. An example of the Stokes

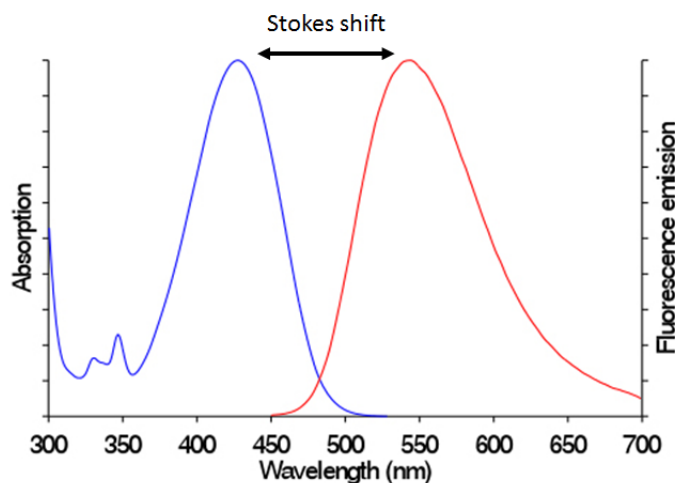


Figure 1.2: Absorption and fluorescence emission spectra of lucifer yellow CH in water.

shift between the excitation and emission light is shown in Fig. 1.2*.

The fluorescence lifetime τ of a molecule is defined as the average time between the absorption of an excitation photon and the subsequent fluorescence emission. It is also defined as the average time a molecule spent in the excited state. Typical values of τ range from less than one nanosecond to more than one millisecond depending on the fluorescent molecules. τ is a quantity that is derived from the population distribution (of excitation-emission intervals) obtained in numerous decay processes, be it measured on identical molecules or in bulk measurements (numerous molecules). The probability density function for this variable is a single exponential decay. We usually observe this for an ensemble of identical molecules or by repeatedly exciting one molecule. The relation between the fluorescence intensity and time shown in Fig. 1.3 can be described in Eq. (1.1) [3, 4]:

$$I(t) = I_0 \exp(-t/\tau) \quad (1.1)$$

where t is time and I_0 is the initial fluorescence at $t = 0$.

When multiple fluorescent species are present, the fluorescence decay will contain a weighted sum of exponential decays. The fluorescence intensity with respect to time for a mixed ensemble of molecules can be described in Eq. (1.2) [3–5]:

$$\sum_i I(t) = I_{0i} \exp(-t/\tau_i) \quad t > 0 \quad (1.2)$$

where τ_i is the lifetime of i th component and I_{0i} is the amplitude of this component which is related to the relative concentration of the component. If photo physical processes occur,

*Image source: <http://www.invitrogen.com/1/1/2805-n-2-aminoethyl-4-amino-3-6-disulfo-1-8-naphthalimide-dipotassium-salt-lucifer-yellow-ethylenediamine.html>. 23 Nov, 2012.

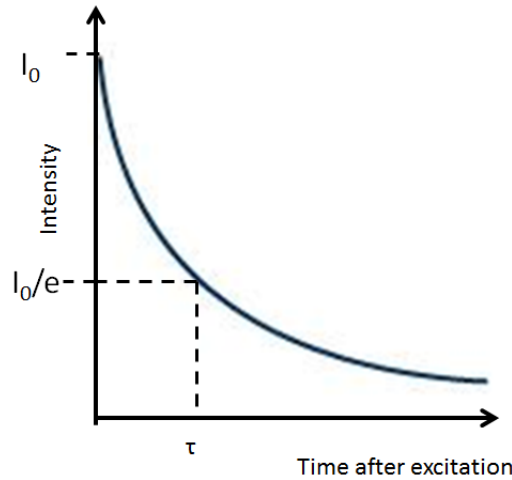


Figure 1.3: An illustration for a single fluorescence decay process.

the observed decay times correspond to the eigenvalues of the collective (interrelated) decay processes and thus do not correspond to the decay of a single chemical species. In addition, in some cases (fluorescence resonance energy transfer included) the reactions are not first-order in character and thus lead to non-exponential decays.

1.2 The importance of FLIM to cell biology research

In cell biology, fluorescence lifetime can be measured using fluorescence lifetime imaging microscopy (FLIM), a technique that involves a fluorescence microscope. Fluorescence microscopy is one type of specialized optical microscopy. The relevant principles of optical microscopy and fluorescence microscopy will be discussed in chapter 2. The techniques that are used to measure fluorescence lifetime are discussed in chapter 3.

The fluorescence lifetime is an intrinsically important biomolecular indicator, which has application in cell biology and cellular pathology. Each type of fluorescence molecule in a specific environment has an average relaxation time after being excited. The fluorescence lifetime is an accurate indicator of available relaxation pathways for each molecule and its environment. Fluorescence lifetime, unlike fluorescence intensity, is not affected by the variation in fluorophore concentrations, static quenching, excitation intensity, and is a robust and reliable fluorescence parameter for characterization of fluorescence species [6]. For example, it can be used to distinguish two fluorophores with similar excitation and emission spectrums but different fluorescence lifetimes. Fluorescence lifetime can also be used to indicate a change in the molecule's environment or the interaction between molecules. For example, the fluorescence lifetime can change in the presence of oxygen or ions [7, 8], changes in local pH [9], and interactions between proteins in living cells [10, 11] etc. Several applications of fluorescence lifetime are discussed below.

Dynamic quenching

Quenching is a process which reduces fluorescence intensity. It can occur in the ground state due to the formation of complexes of molecules (static quenching) or during the excited state (dynamic quenching). In the dynamic quenching process, the excited molecules will accelerate their relaxation to the ground state with the assistance of collisional quenchers present in the environment, such as triplet oxygen [12], Br^- [13], I^- [14, 15], Cs^+ [16] and acrylamide [6, 14, 17]. The result of the dynamic (collisional) quencher is that the fluorescence lifetime is shortened. Since in this case it is not certain whether the decreased fluorescence intensity is due to reduction in the number of fluorophores or static quenching (no change in lifetime) or dynamic quenching (lifetime reduced), fluorescence lifetime is a very suitable tool to determine accurately dynamic quenching rates. In the case of dynamic quenching, the relationship of the fluorescence lifetime and the quenching rate can be given as Eq. (1.3) [6]:

$$\frac{\tau^-}{\tau^+} = 1 + k\tau^- \quad (1.3)$$

where τ^- is the lifetime measured with the absence of the quencher and τ^+ is that with the quencher; k is the quenching rate.

Föster resonance energy transfer

One of the major applications of FLIM is Föster resonance energy transfer (FRET). FRET is a process where energy transfer occurs while a donor molecule is in the excited state. If the excitation spectrum of the acceptor overlaps the emission spectrum of the donor, the donor chromophore can transfer its energy to an acceptor chromophore through nonradiative dipole-dipole coupling. The distance between donor and acceptor must be very small ($< 10\text{nm}$). The principle of FRET is shown in Fig. 1.4. The FRET efficiency is inversely proportional to the sixth power of the distance between donor and acceptor and can be used as an effective ruler to measure this distance. FRET does not require the acceptor chromophore to be fluorescent, but in most cases both the donor and the acceptor are fluorescent. To measure the FRET efficiency, the fluorescence intensity signal with and without the presence of the acceptor must be compared. Since the variability of the concentrations of fluorophores in the biological cells is unknown, it is difficult to quantify FRET using steady-state fluorescence. With fluorescence lifetime, however, there is no intensity calibration step involved. One only needs to know the fluorescence lifetime of the donor with and without the presence of the acceptor, as shown in Eq. (1.4)[6].

$$E_{FRET} = \frac{1}{1 + \left(\frac{R}{R_0}\right)^6} = 1 - \frac{\tau_D^{+A}}{\tau_D^{-A}} \quad (1.4)$$

where R is the distance between two centers of the donor and acceptor fluorophores, R_0 is the distance of this donor and acceptor pair at which the energy transfer efficiency is 50%, τ_D^{+A} and τ_D^{-A} are the donor fluorescence lifetimes in the presence and absence of the

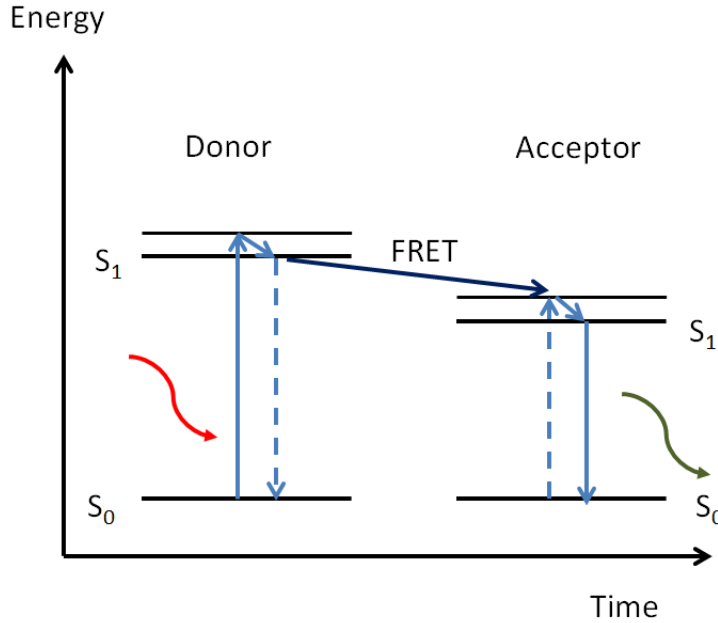


Figure 1.4: Jablonski diagram of FRET.

acceptor, respectively. The Eq. (1.4) is applicable when the quantum yield of the donor is not affected by the physical-chemical consequences of the complex formation itself (e.g. by changes in polarity).

Anisotropy

Fluorescence anisotropy is a measure of fluorescence emission polarization, which can provide valuable information about the binding constants and reaction kinetics, which change the rotational time of the molecules. The rotational correlation time ϕ_{rot} , the fluorescence lifetime τ_F and the steady-state anisotropy of the molecule $r_{steady-state}$ can be related as in Eq. (1.5)[6]:

$$r_{steady-state} = r_0 \frac{1}{1 + \tau_F / \phi_{rot}} \quad (1.5)$$

where r_0 is a limiting number given by the relative orientation of the excitation and emission transition dipoles. By knowing the $r_{steady-state}$ and τ_F , one can assess the rotational correlation time ϕ_{rot} , which gives profound information about the molecular environment of the fluorescence molecule [18, 19]. With the knowledge of τ_F , which can be measured from FLIM and ϕ_{rot} , the effective viscosity of the solvent surrounding the molecule can be studied.

Each of the three examples above - quenching, FRET and anisotropy - shows that fluorescence lifetime can provide directly accessible biophysical information about cellular processes.

1.3 Aim and thesis outline

It is clear from the above applications that FLIM is a sophisticated tool. To measure fluorescence lifetime there are two “standard” approaches - one is the time domain and one is the frequency domain.

The work of this thesis was carried out as part of the MEM-FLIM (Modulated Electron Multiplied all-solid-camera for Fluorescence Lifetime Imaging Microscopy) project. In this project, we have designed, built, and tested an all-solid-state frequency-domain FLIM system which has a better performance than the currently intensifier-based frequency-domain FLIM system. Besides Delft University of Technology, three other partners have been involved in this project: Lamberts Instruments in Roden, the Netherlands Cancer Institute in Amsterdam, and Teledyne DALSA in Eindhoven. The aim of this thesis is to describe the development of the modulated electron multiplied all-solid-camera for fluorescence lifetime imaging microscopy and the principle and performance of our FLIM system, from theory to practical experiments.

The basic structure of the thesis outline is as follows:

Chapter 2: Fluorescence lifetimes are measured quantitatively using fluorescence lifetime microscopy (FLIM) techniques. FLIM is a technique developed and based on fluorescence microscopy. In order to understand FLIM, we will start the discussions from basic optical microscopy and proceed to fluorescence microscopy.

Chapter 3: After understanding the basic principle of the fluorescence lifetime and its important usage in biology research which is discussed in the first chapter, with the knowledge of optical microscopy and fluorescence microscopy (chapter 2), we will describe how we are going to measure the fluorescence lifetime in this chapter.

Chapter 4: The image sensor is a crucial element in a FLIM system. In the current frequency-domain FLIM, the image intensifier also plays an important role. In the MEM-FLIM project, however, we are building special image sensors which eliminate the use of the image intensifier in the frequency-domain FLIM. Thus it is of importance to understand the principle of the image sensors and the strengths and weaknesses of the image intensifier. A technical description of the image sensors (charge-coupled devices) and as appropriate image intensifiers are discussed.

Chapter 5: A mathematical model is constructed to analyze the photon efficiency of fluorescence microscopy. This is a necessary preparatory step for building a novel FLIM system. The power of the light source needed for illumination in a FLIM system and the signal-to-noise ratio (SNR) of the detector are determined. One can thus have a better understanding of the optical signal flow and its loss in the electro-optical system. In this sense, we have named this chapter as “Photon Budget”.

Chapter 6: Novel all-solid-state directly modulated CCD cameras have been devel-

oped to improve current intensifier-based CCD camera in frequency domain FLIM. In this chapter, two architectures will be introduced. One is the horizontal toggling MEM-FLIM camera (for simplicity, we name this “MEM-FLIM1” camera), and one is the vertical toggling MEM-FLIM (“MEM-FLIM2”) camera. The operational principles of MEM-FLIM1 and MEM-FLIM2 are discussed in this chapter.

Chapter 7: Definition of camera performance indicators, such as dark current, sensitivities, etc. are presented in this chapter, followed by the camera evaluation methods used to compare the MEM-FLIM cameras with a reference camera.

Chapter 8: Camera characteristics of MEM-FLIM(1,2) and the reference camera such as noise distribution, dark current influence, camera gain, sampling density, sensitivity, linearity of photometric response, and optical transfer function etc. have been studied through experiments. Lifetime measurement using our MEM-FLIM (1,2) camera for various objects are discussed, e.g. fluorescein solution, fixed GFP cells, and GFP-Actin stained live cells. A detailed comparison between a conventional micro-channel plate (MCP)-based FLIM system and the MEM-FLIM system is presented, together with the comparison between MEM-FLIM camera and another all-solid-state FLIM camera.

Chapter 9: Based on the evaluations of the MEM-FLIM1 and MEM-FLIM2 systems, the architecture of the MEM-FLIM camera has been updated to the version MEM-FLIM3, which is discussed in this chapter. Compared to the first design (MEM-FLIM1 and MEM-FLIM2), MEM-FLIM3 has architectural advantages such as larger pixel number, higher modulation frequency, etc.

Chapter 10: Evaluations of MEM-FLIM3 are discussed in this chapter. The same methods used to evaluate MEM-FLIM(1,2) are employed to characterize MEM-FLIM3.

CHAPTER 2

Fluorescence Microscopy

Abstract

Since fluorescence lifetime imaging microscopy (FLIM) is a technique developed and based on optical (fluorescence) microscopy, we need first to understand the basics of optical microscopy, and then fluorescence microscopy in order to understand FLIM. In this chapter, technical aspects of optical microscopy, in particular fluorescence microscopy are presented. Illumination techniques, important elements in optical microscopy such as the light sources and the objective lenses are discussed. For fluorescence microscopy, comparison between wide-field microscopy and confocal microscopy are discussed. Different types of fluorescent samples are presented. Photobleaching, one of the limitations of fluorescence microscopy, is also discussed.

Keywords: optical microscopy, fluorescence microscopy, illumination technique, light source, objective lens, fluorescence sample, photobleaching

2.1 Optical microscopy

2.1.1 Introduction and history

Microscopy is the technical field of magnifying and viewing samples which are below the resolution range of unaided eyes. Optical microscopy, also referred as “light microscopy”, employs visible light and a set of optical elements (lenses, filters) to image the small objects.

The first compound microscope was built by Zacharias Jansen and his son Johannes around 1590 [20, 21]. This microscope consisted of two lenses, an objective lens close to the sample and an eyepiece, and managed to do two-stage magnification. Antonie Philips van Leeuwenhoek improved the microscope, enhanced the magnification to 266 times by using high quality optical lenses. He was the first to observe and describe the single-celled organisms, and was known as “the Father of Microbiology” due to his great discoveries such as muscle fibers, bacteria, spermatozoa, protozoa, and blood flow in capillaries, etc. [22, 23]. The images produced by these early microscopes suffered from aberrations. The development of achromatic objectives in the mid-nineteenth century by Joseph Lister and Giovanni Amici reduced chromatic aberration and increased numerical apertures [20]. Ernst Abbe’s mathematical theory on the limitation of resolution of an optical microscope [24] and his collaboration with Carl Zeiss led to great success in a theoretical and technical view of microscopy. Images free of chromatic aberration and reduced spherical aberration were obtained using advanced objective lenses based upon their achievements. Several years later, in 1893, August Köhler introduced an illumination method, allowing the illumination to take full advantage of the resolving power of the objective lens [25]. In 1930s, Dutch physicist Fritz Zernike developed the technique called “Phase contrast microscopy”, for which he was later awarded the Nobel Prize [26]. Transparent samples such as live mammalian cells were able to be imaged without staining by using interference instead of absorption of light. Differential interference contrast (DIC) microscopy was developed by Georges Nomarski in 1955 [27]. Lots of specialized light microscopy techniques were developed in the twentieth and twenty-first century such as interference reflection microscopy (RIC), fluorescence microscopy, confocal microscopy, single plane illumination microscopy, fluorescence lifetime imaging microscopy (FLIM), stimulated emission depletion microscopy (STED) and Structured Illumination Microscopy (SIM).

2.1.2 Illumination techniques

A bright, glare-free and even illumination is a key element to produce high quality images in optical microscopy. One of the most frequently used methods is Köhler illumination [28–30], the main advantages of which are evenly distributed illumination and high contrast. Köhler illumination was introduced by August Köhler and Carl Zeiss in 1893 and requires (1) a collector and/or field lens, which collects and focuses the light from the light source at the condenser diaphragm plane; (2) a field diaphragm which can adjust the amount of light entering the sample; (3) a condenser diaphragm which changes

sample contrast; and (4) a condenser lens which projects the light through the sample without focusing it.

Before Köhler illumination was introduced, critical illumination was the predominant technique [29–31]. The disadvantage of critical illumination is its uneven illumination: the image of the light source falls in the same plane as the object instead of the condenser diaphragm plane as in Köhler illumination. Critical illumination has been largely replaced by Köhler illumination in modern scientific optical microscopy.

2.1.3 Light sources

Early optical microscopes used natural sunlight or oil lamps as their light source. Even though the microscopists tried to gather the light in many ways, these type of light sources could not provide reliable illumination and often caused glare or flooding. Modern microscopes, however, have their own controllable light sources. One of the main light sources is incandescent tungsten-based lamps such as tungsten-halogen lamps. They consist of a glass bulb filled with inert gas and a tungsten wire filament. The shape of the glass bulb, the filament arrangement and the mounting fixtures may vary. These lamps provide a continuous spectrum from about 300 nm to 1400 nm, and the majority of the wavelength intensity is in the 600-1200 nm region [32]. Compared to other tungsten-based lamps, tungsten-halogen lamps have advantages such as smaller size, uniform illumination and longer lifetime.

Arc lamps (mercury, xenon and zirconium arc lamps) are used in specialized microscopy such as fluorescence microscopy. These lamps are gas discharge tubes filled with metal gas with an average lifetime around 200 hours. The intensity peaks of the mercury arc lamp are at 313, 334, 365, 406, 435, 546, and 578 nm [32]. The continuous spectrum between the near-ultraviolet to near-infrared produced by xenon arc lamps closely mimics natural sunlight. A large proportion of the xenon arc lamp spectrum is in the infrared, which makes heat control necessary, and they are deficient in the ultraviolet range.

Lasers are also a popular light source in modern microscopy techniques such as fluorescence microscopy, fluorescence lifetime imaging microscopy, scanning confocal microscopy, monochromatic bright field microscopy, etc. They can provide high intensity light with a very narrow spectrum. The disadvantages of lasers are their high cost and the “speckle” effect caused by laser coherence.

Light emitting diodes(LED) are becoming increasingly popular in wide-field fluorescence (lifetime imaging) microscopy. Their low cost (compared to lasers), lower heat generation, long lifetime (compared to Arc lamps) and emission in a variety of colors enable them to enter the scientific research market. Figure 2.1 is an example of spectra of some common light sources, including a tungsten lamps, a mercury lamp, a white LED, a bar code scanning-laser and sunlight at noon*. In this thesis, the fluorescence lifetime experiments are mainly carried out using LED as the light source.

*Image source: <http://www.olympusmicro.com/primer/lightandcolor/lightsourcesintro.html>. 21 March, 2013

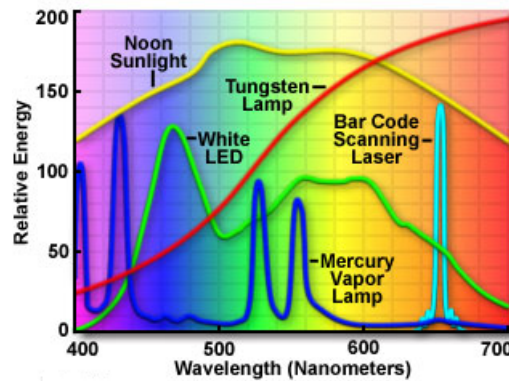


Figure 2.1: Spectra from some common light sources.

2.1.4 Objective lenses

For an optical microscope, the most difficult element to design is the objective lens. Objective lenses are responsible for gathering the light from an object and forming the primary image. The image quality and the magnification depend heavily on the quality and parameters of an objective lens. The objective lens is usually a cylinder containing one or more lenses. Some important parameters for an objective lens are:

- Numerical Aperture (NA): Numerical aperture, expressed as $NA = n \sin \theta$ describes the ability of the lens to collect light. θ is the “acceptance angle” of the lens, and n is the index of refraction of the immersion medium of the lens. The physical size of the lens contributes to the NA value of a lens and the light should completely fill the back focal plane of the objective in order to get the most out of it. NA plays a central role in determining the resolving power of a lens. A lens with a higher NA has a higher resolving power, as shown in Eq. (2.1), and the image it can produce is brighter.

$$\text{Resolution} \propto \frac{1}{NA} \quad (2.1)$$

- Magnification (M): The magnification measures the enlargement of the sample image. Together with the NA, the magnification controls the brightness of an image. For Köhler illumination: the image brightness is proportional to the square of the NA and inversely proportional to the square of the M: $\text{ImageBrightness} \propto NA^2/M^2$; while for the critical illumination, the image brightness is proportional to the 4th power of the NA and inversely proportional to the square of the M: $\text{ImageBrightness} \propto NA^4/M^2$.
- Immersion medium: The light collecting ability of an objective not only depends on NA, but also depends on the medium through which the light travels. Different media have different refractive indices n . Most microscope objectives use air ($n = 1$)

as the medium and they are often referred as dry objectives. Some also use water ($n = 1.33$), glycerine ($n = 1.47$) or immersion oils (average $n = 1.51$). The advantage of using an objective designed with immersion oil compared to those that are used dry is that immersion objectives are typically of higher correction (either fluorite or apochromatic) and can have working numerical apertures up to 1.40 (dry objectives can produce an NA up to 0.95). These objectives allow opening of the condenser diaphragm to a greater degree and take advantage of the increased NA.

- Depth of field (DOF): The axial distance over which the sample is in focus is called the depth of field of an objective [33], which is described in Eq. (2.2). λ is the wavelength. A higher NA leads to a higher resolving power but a smaller DOF.

$$DOF = \frac{\lambda}{2NA^2} \quad (2.2)$$

2.1.5 Resolution limitations

The diffraction of a point source is an indicator of the quality of an image system, since the imaged point source will not be the same as the original due to the diffraction of the transmitted light. The diffraction pattern of a point light source has a brighter region in the center which is called the Airy disk, together with a pattern of concentric bright rings around it, the Airy pattern. This diffraction pattern is characterized by the wavelength of light source and the objective aperture's size. The point spread function (PSF) mathematically describes the Airy disk of a point source, the intensity of the Airy pattern is characterized in Eq. (2.3)[34]:

$$psf(r) = \left[2 \frac{J_1(ar)}{r} \right]^2 \quad (2.3)$$

where $a = 2\pi NA/\lambda$, λ is the wavelength of the illumination light, NA is the numerical aperture of the objective, J_1 is the Bessel function of the first kind of order one, and r is the radius distance. The PSF and the size of the Airy disk depend on the NA of the objective and the wavelength of the illumination. A higher NA results in a higher resolving power (a smaller Airy disk).

The resolution, which can be measured by the size of Airy disk, is defined as the minimum distance at which two objects can be resolved. There are two closely related values for the diffraction limit, the Rayleigh and Abbe criterions; the difference between them is not large in practical applications.

Lord Rayleigh gave a criterion for the minimum distance between two Airy disks that can be resolved in Eq. (2.4) [33]:

$$d_r = \frac{0.61\lambda}{NA} \quad (2.4)$$

By using this equation, for example, one can determine the smallest distance that can be resolved by an optical microscope to be around 218 nm given $NA = 1.4$ and $\lambda = 500$ nm.

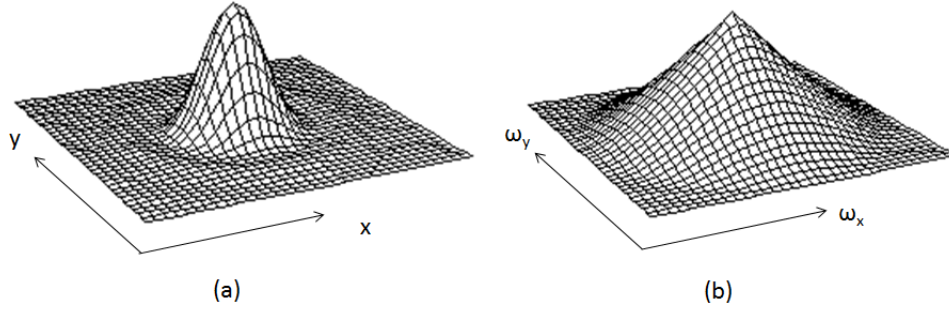


Figure 2.2: An illustration of PSF and OTF. (a) 2D PSF displaying an Airy structure, (b) 2D OTF for a diffraction-limited lens.

The Abbe diffraction limit offers an alternative approach to determine the resolution of an optical system, as shown in Eq. (2.5) [35]. Abbe took the coherence into account while Rayleigh assumes the light is incoherent. By using this equation, for example, one can determine the smallest distance that can be resolved by an optical microscope to be around 179 nm given $NA = 1.4$ and $\lambda = 500$ nm.

$$d_a = \frac{\lambda}{2NA} \quad (2.5)$$

The optical transfer function (OTF), which is the Fourier transform of the PSF, is quite often used to describe the resolution. For an idea circularly-symmetric, diffraction-limited objective, the OTF is shown in Eq. (2.6) [36]:

$$OTF(f) = \begin{cases} (2/\pi) \left(\arccos(f/f_c) - (f/f_c)\sqrt{1 - (f/f_c)^2} \right) & |f| \leq f_c \\ 0 & |f| > f_c \end{cases} \quad (2.6)$$

where f is the radial distance in the frequency plane and the cutoff frequency $f_c = 2NA/\lambda$. $OTF(f = 0) = 1$, indicating no intensity is lost as light goes through the lens. Figure 2.2[†] shows an illustration of PSF and OTF. Note the circular-symmetry in both the PSF and OTF. The OTF describes the axial performance of a lens system and its absolute value defines contrast and spatial bandwidth.

2.2 Fluorescence microscopy

2.2.1 Techniques

One of the specialized optical microscopy techniques is fluorescence microscopy. Fluorescence microscopy concerns any microscope that uses fluorescence to generate an image. Fluorescence microscopy can be a simple technique such as Epi-fluorescence [37] or

[†]Image source:[34].

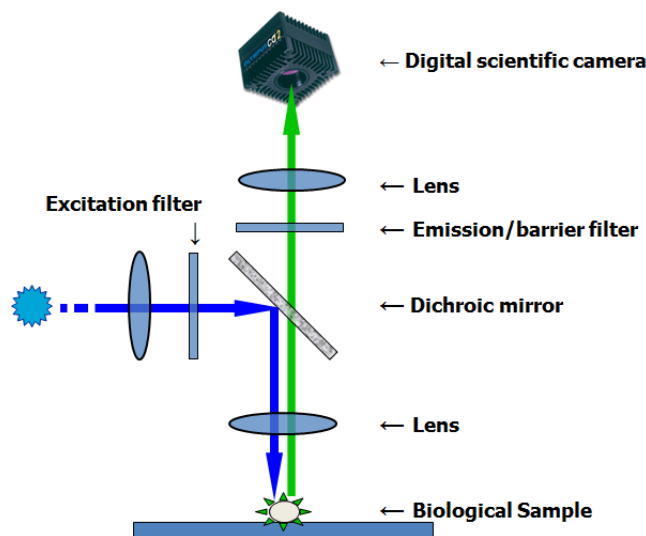


Figure 2.3: The basic diagram of an epifluorescence fluorescence microscope.

a more complex technique such as confocal laser scanning microscopy (CLSM) [38, 39], 4π -microscopy [40], two-photon microscopy [41], theta-microscopy [42], or total internal reflection fluorescence microscopy (TIRF)[43].

The basic diagram of a conventional wide-field (WF) epifluorescence microscope is shown in Fig. 2.3. The sample of interest is illuminated through the lens with higher energy (shorter wavelength) photons. This causes the sample to emit lower energy (longer wavelength) photons. The filter sets and dichroic mirror are configured so that only the desired emission light will reach the eyepiece or the detector. In epi-illumination, the excitation light and the sample emission light pass through the same objective lens.

In the WF fluorescence microscope, the entire specimen is “bathed” in the excitation light and the resulting fluorescence emission is collected by the detector. The fluorescence emission from the specimen which is not in the plane of focus often interferes with those that are in focus. To overcome this problem, confocal microscopy was invented. The objective lens focuses the excitation light at the desired focal plane, and a second pinhole before the detector allows only the in-focus emission to pass and reach the detector. In this way, the optical resolution and the contrast, especially in the axial (depth) direction, can be improved compared to the WF microscope. The light pathways in confocal microscopy are shown in Fig. 2.4[‡].

Both the confocal microscope (CM) and the WF microscope have their advantages. The optimal usage of WF microscopy is for studying thin sparsely stained specimens. By rejecting out-of-focus emission light, the CM can yield a better lateral and axial resolution for thick specimen. The CM can be regarded as a serial device: 2D and 3D images can be acquired by applying scanning technique since only one focal plane in the sample is

[‡]Image source: http://serc.carleton.edu/microbelife/research_methods/microscopy/fluomic.html. 6 Nov 2012.

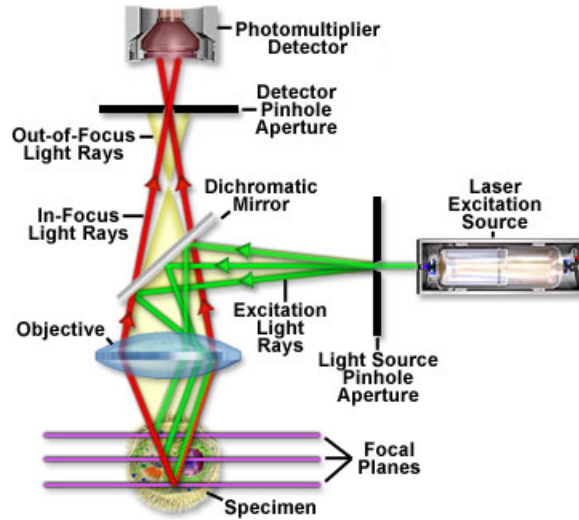


Figure 2.4: Principle light pathways in confocal microscopy.

illuminated at one time. One particular embodiment of the CM is the confocal laser scanning microscope (CLSM) which, using a laser excitation source and a galvanometer-driven mirror, scans a given focal plane on a point-by-point basis. The WF microscopy, however, can be treated as a parallel device since all pixels in the image are recorded simultaneously. This allows the WF microscope to have a higher image acquisition rate compared to the CLSM.

2.2.2 Fluorescent samples

Fluorescence microscopy, which utilizes fluorescence emission light to observe the sample structure, requires some preparation for the sample. The main technique to prepare a fluorescent sample in biological samples is to label the sample with fluorophores or expression of fluorescent protein. Alternatively the intrinsic fluorescence of a sample can be used, e.g. NADPH [44] or flavins [45].

Fluorophores are chemical compounds that exhibit fluorescent properties. They can be used as a tracer in fluids, or as a dye for staining biological structures, or as a probe or indicator. Most fluorophores are of the size of 20-100 atoms. There are many fluorescent reporter molecules such as DAPI, fluorescein, derivatives of rhodamine (TRITC), coumarin, and cyanine. In this thesis, fluorescein and rhodamine 6G will often be used to calibrate an FD-FLIM system before a lifetime of an unknown sample is measured.

In cell and molecular biology, DNA can be genetically modified so that a fluorescent protein reporter can be carried. The fluorescent protein can be used as a biosensor such as in FRET experiments. The most frequently used proteins are GFP (green fluorescent protein), RFP (red fluorescent protein), CFP (Cyan fluorescent protein), YFP (yellow fluorescent protein), and their derivatives [11, 46–50]. The discovery of GFP made it possible for biologists to look into the living cell for the first time.

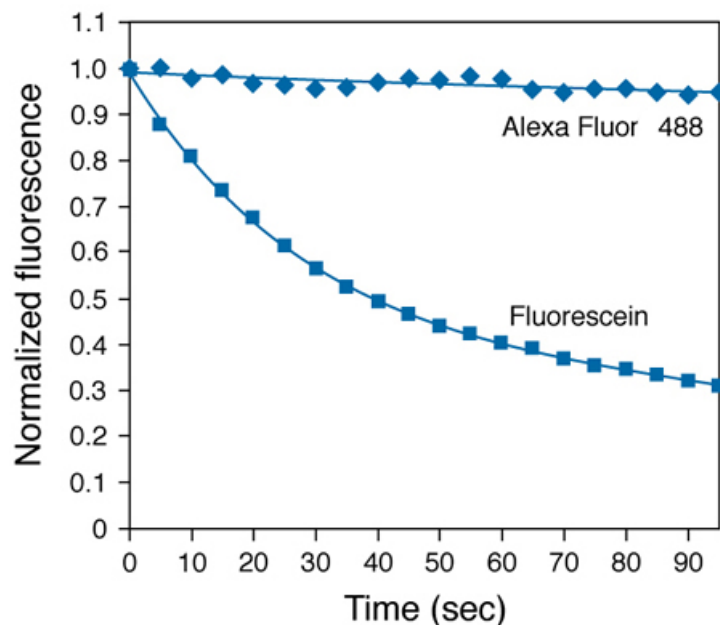


Figure 2.5: An illustration of photobleaching of Fluorescein and Alexa Fluor448 over time.

Some other fluorescence particles such as quantum dots (2-10 nm diameter, 100-100,000 atoms) can be also used in fluorescence microscopy [51, 52].

2.2.3 Limitations

A fluorophore generally suffers from a photochemical destruction called photobleaching [53]. Fluorophores lose their ability to fluorescence as they are being illuminated. This photobleaching rate varies for different fluorophores. Photobleaching may complicate and limit the observation of a fluorescent sample. This causes trouble in intensity-based measurement and especially in time-lapse microscopy. For this reason biologists avoid the use of long-term, high intensity illumination. Figure 2.5[§] shows an example for fluorescein and Alexa Fluor448 bleaching over time.

Photobleaching, however, can also be used to study motion or molecule diffusion such as in FRAP (Fluorescence Recovery After Photobleaching) and FLIP (Fluorescence Loss In Photobleaching) techniques. In some cases signal-to-noise ratios can be improved by intentionally using photobleaching to irradiate autofluorescence.

[§]Image source: <http://www.invitrogen.com/site/us/en/home/support/Research-Tools/Image-Gallery/Image-Detail.8391.html>. 21 March 2013.

2.3 Summary

The aim of this chapter is to provide the necessary background information for this thesis, the principles associated with the MEM-FLIM system. It starts with an introduction to optical microscopy, its basic elements such as illumination method, commonly used light sources, objective lenses, and the concept of the diffraction and resolution limitation. The specialized technique- fluorescence microscopy- is presented and discussed.

Fluorescence lifetime imaging microscopy

Abstract

In this chapter, technical aspects of FLIM are presented, in particular the frequency-domain version. Two approaches of measuring fluorescent lifetime (time-domain FLIM and frequency-domain FLIM) are discussed. We focus more on frequency-domain method since MEM-FLIM cameras are developed for such systems.

Keywords: fluorescence lifetime, fluorescence lifetime imaging microscopy (FLIM)

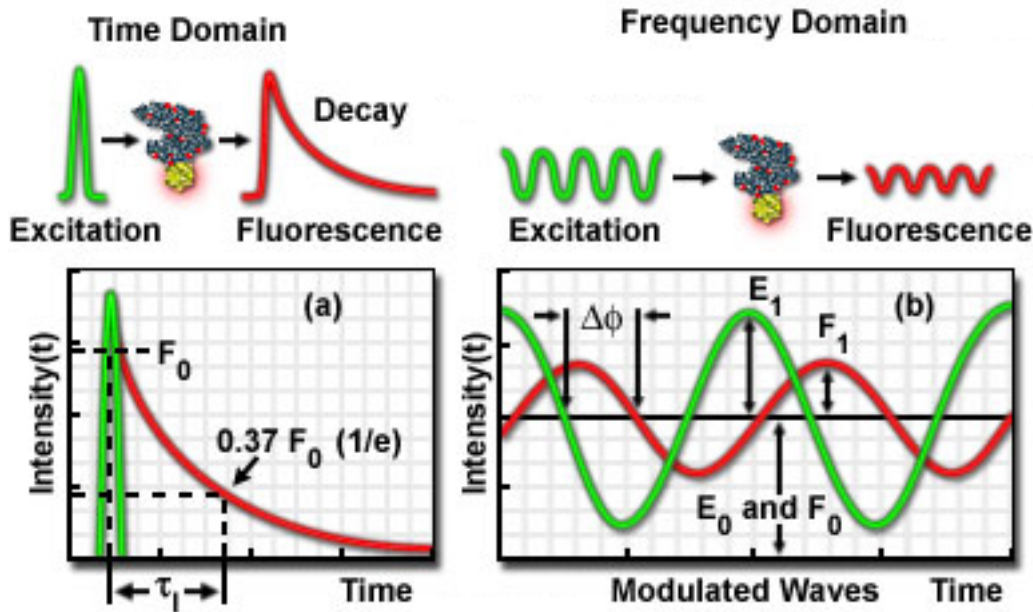


Figure 3.1: Two methods of fluorescence lifetime imaging: the time-domain method and the frequency-domain method.

Fluorescence imaging methods can provide a wealth of information about biology samples. Besides fluorescence intensity measured, one of the most important indicators is the fluorescence lifetime, which can be measured by fluorescence lifetime imaging microscopy (FLIM) techniques. Instrumental methods for measuring fluorescence lifetime can be divided into two major categories: time domain (TD) and frequency domain (FD), as shown in Fig. 3.1*. Fluorescence lifetime of typical dyes is in 0.5-20 ns range [54].

3.1 TD-FLIM

In TD-FLIM, a train of pulsed light, where the width of each pulse should be significantly smaller than the decay time of the fluorescent sample, is used for excitation. The decay curve of the emission photons is detected using a time-resolved detection system [55–57]. It is an inherently direct measurement of the fluorescence decay. The data analysis of TD-FLIM is typically achieved by fitting the experimental data to a linear combination of decaying exponentials, as shown in Eq. (3.1). A typical value of a laser light pulses is 50 ps full width at half maximum (FWHM) with a repetition rate of up to

*Image source: <http://www.olympusfluoview.com/applications/flimintro.html>. 8 Nov, 2012.

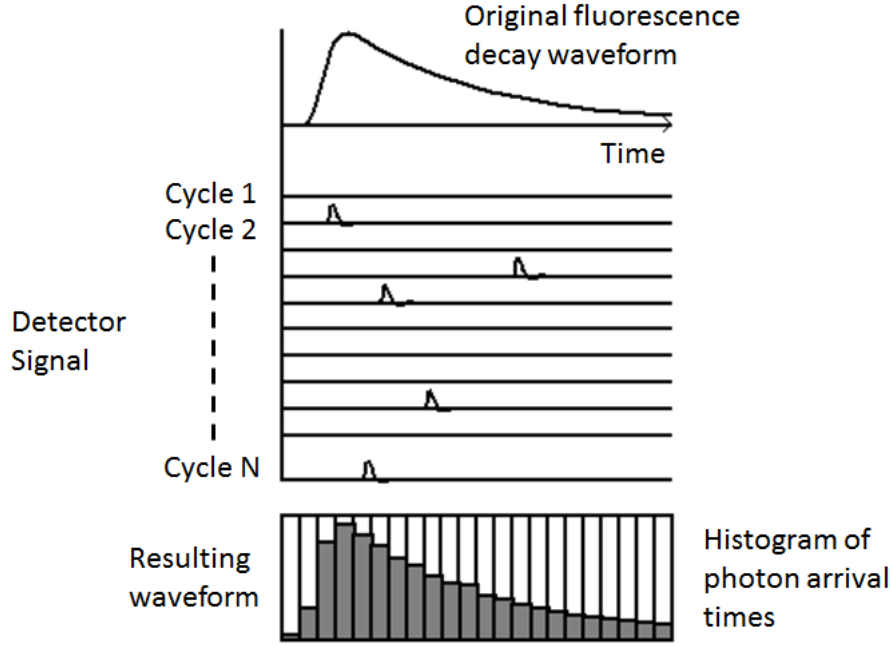


Figure 3.2: The principle of the TCSPC.

80 MHz, the shortest lifetime can be measured is around 10ps [58].

$$I(t) = \sum_k p_k \exp\left(-\frac{t}{\tau_k}\right) \quad t \geq 0 \quad (3.1)$$

The values of τ_k represent the different lifetime components in the sample under study and the values of p_k are their relative contributions. The fitting process not only costs computation time but generally requires a high level of expertise to obtain reliable results [59]. The TD-FLIM system is also relatively expensive since it requires short pulsed lasers and fast, sensitive detection systems.

One well-known method in TD-FLIM is time-correlated single photon counting (TCSPC) [60–63], which is based on measuring the average time of the first arriving photon after the sample is excited. A high repetitive rate mode-locked picosecond or femtosecond laser light source is needed and a single photon sensitive detector, such as a photomultiplier tube (PMT) or a single photon avalanche diode (SPAD) can be used. The histogram of photon arrival times represents the time decay one would have obtained from a “single shot” time-resolved recording assuming a low possibility of registering more than one photon per cycle [64]. The TCSPC is perfectly compatible with CLSM and the sample is scanned in order to obtain a 2D or 3D image. The principle of the TCSPC is shown in Fig. 3.2. Another well-known method in TD-FLIM is time gated FLIM [65–67], which can be implemented not only on CLSM but also on WF microscopy. The principle is shown in Fig. 3.3. In this method, a pulsed excitation is employed. The fluorescence emission is

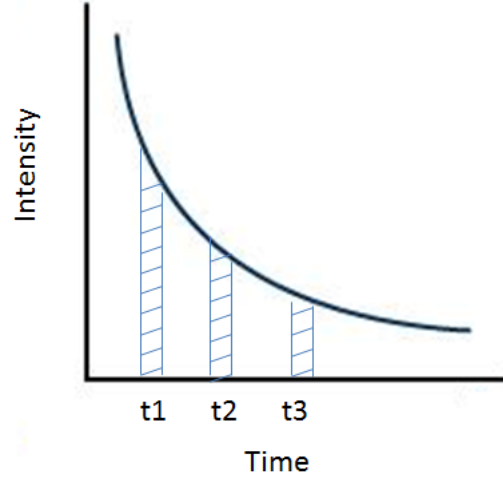


Figure 3.3: The principle of time gated FLIM.

detected sequentially in two or more time gates each delayed by a different time relative to the excitation pulse [65]. The ratio of the obtained fluorescence signal is a measure of fluorescence lifetime in the case of two gates of equal width for a mono-exponential decay. Increasing the number of gates enables the calculation for multi-exponential decays. The disadvantage of this method is its low photon efficiency since only part of photons are recorded, which leads to a longer sample exposure and the problems associated with photobleaching as described above.

3.2 FD-FLIM

3.2.1 Theory and mathematical model

Instead of measuring the fluorescence lifetime in the time domain, an alternative way is through the frequency domain approach FD-FLIM. FD-FLIM uses periodically modulated light for the excitation and estimates the lifetime values from the phase change and/or the modulation depth change between excitation and emission signals. For the fluorescence molecules with the same lifetime, the average response after the excitation is derived from Eq. (3.1) and given by:

$$fluorescence(t) = \frac{1}{\tau} e^{-\frac{t}{\tau}} \quad t \geq 0 \quad (3.2)$$

The excitation with a zero phase is defined as Eq. (3.3).

$$excitation(t) = 1 + m_{excitation} \sin(\omega t) \quad m_{excitation} \leq 1 \quad (3.3)$$

The modulation depth m is defined as 1/2 of the peak-to-peak intensity value divided by the DC intensity value. For example, in the case of the excitation, $m_{excitation} = E_1/E_0$, E_0 is the excitation DC intensity value, and E_1 is the 1/2 of the peak to peak excitation intensity value, as shown in Fig. 3.1. The modulation depth m of both excitation and emission should be smaller than one since there is no negative light. ω is the angular frequency of the modulation.

Ignoring the signal amplitude change, the resulting emission is the convolution of the excitation and fluorescence response. Since the fluorescence response is modeled as a linear, time-invariant system, the emission will be in the form of Eq. (3.4):

$$emission(t) \propto excitation(t) \otimes fluorescence(t) \propto 1 + m_{emission} \sin(\omega t - \theta) \quad m_{emission} \leq 1 \quad (3.4)$$

where θ is the phase change introduced by the fluorescence response. The ratio of the modulation depth of the emission signal to that of the excitation signal m is defined as $m = m_{emission}/m_{excitation}$. The θ and m can be calculated from Eq. (3.4), as shown in Eq. (3.5) and Eq. (3.6):

$$\theta = \arctan(\omega\tau) \quad (3.5)$$

$$m = \frac{1}{\sqrt{(\omega\tau)^2 + 1}} \quad (3.6)$$

In another words, by measuring the phase delay and the ratio of the modulation depth of the emission signal to that of the excitation signal, the fluorescence lifetime can be calculated, as shown in Eq. (3.7) and Eq. (3.8):

$$\tau_\theta = \frac{1}{\omega} \tan(\theta) \quad (3.7)$$

$$\tau_m = \frac{1}{\omega} \sqrt{\frac{1}{m^2} - 1} \quad (3.8)$$

A common practice to retrieve the phase and the modulation depth is to demodulate the emission signal with a frequency that is either the same (homodyne method) or close to (heterodyne method) the modulation frequency of the excitation signal [68], the former of which is more commonly used [69–71]. In the homodyne method, the emission signal is multiplied by the demodulation signal on the detector which has phase φ relative to the excitation signal and a modulation depth of the detector's sensitivity $m_{detector}$, as shown in Eq. (3.9). The resulted detection signal is a low-pass filtered signal of the product of emission signal in Eq. (3.4) and detector signal in Eq. (3.9), which is described in Eq. (3.10):

$$detector(t) = 1 + m_{detector} \sin(\omega t - \varphi) \quad (3.9)$$

$$\begin{aligned} detection(t) &= lowpass\{emission(t) \cdot detector(t)\} \\ &= lowpass\{(1 + m_{emission} \sin(\omega t - \theta)) \cdot (1 + m_{detector} \sin(\omega t - \varphi))\} \\ &= 1 + \frac{1}{2} m_{emission} m_{detector} \cos(\varphi - \theta) \end{aligned} \quad (3.10)$$

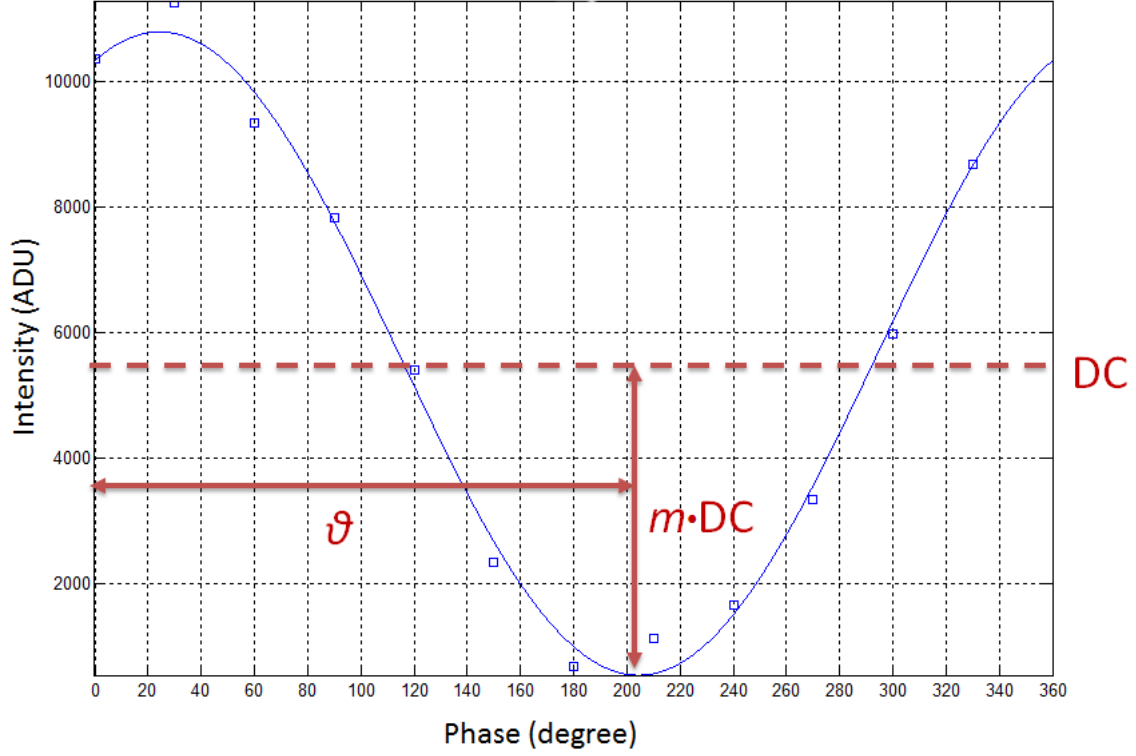


Figure 3.4: An illustration of the homodyne method. Data points from twelve measurements are used to fit a sine function.

By deliberately varying the phase of detector φ , the resulted detection signal intensity at different phase steps can be fitted with a sine function, from which the phase θ and the modulation depth m can be obtained, as shown in Fig. 3.4.

A typical commercially available FD-FLIM system, which is used in this thesis as the reference FLIM system, is shown in Fig. 3.5.

3.2.2 AB plot

For a single fluorescence lifetime system, the lifetime derived from the phase change τ_θ will be the same as that from the modulation depth change τ_m . When the difference between these two derived lifetime values is relatively big, we suspect that the sample contains multiple lifetime decays. The phase change and the modulation depth change

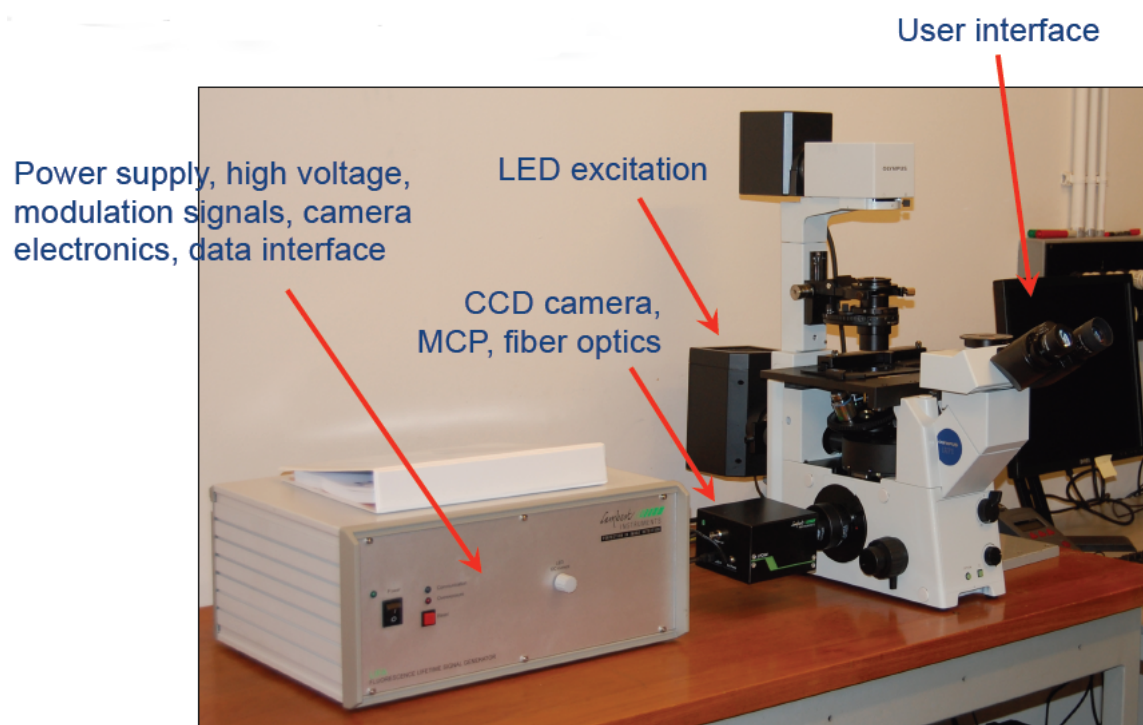


Figure 3.5: The illustration of an typical FD-FLIM system.

for a multi-lifetime system can be described as Eq. (3.11) and Eq. (3.12).

$$\theta = \arctan \left(\frac{\sum_j \frac{\alpha_j \cdot \omega \tau_j}{1 + (\omega \tau_j)^2}}{\sum_j \frac{\alpha_j}{1 + (\omega \tau_j)^2}} \right) \quad (3.11)$$

$$m = \sqrt{\left(\sum_j \frac{\alpha_j \cdot \omega \tau_j}{1 + (\omega \tau_j)^2} \right)^2 + \left(\sum_j \frac{\alpha_j}{1 + (\omega \tau_j)^2} \right)^2} \quad (3.12)$$

The subscript j refers to the j th lifetime component, α_j is its relative contribution, and $\omega = 2\pi f$ is the circular frequency corresponding to the modulation frequency f . By doing lifetime measurements under multiple frequencies, the lifetime components and their contributions can be extracted. An AB plot (a plot of A vs. B), also known as a “phasor plot”, is quite often used to represent lifetime results for a two-lifetime component system [72–74], where A and B are defined in Eq. (3.13) and Eq. (3.14):

$$A_i = m_i \sin(\theta_i) = \frac{\alpha_i \omega \tau_1}{1 + (\omega \tau_1)^2} + \frac{(1 - \alpha_i) \omega \tau_2}{1 + (\omega \tau_2)^2} \quad (3.13)$$

$$B_i = m_i \cos(\theta_i) = \frac{\alpha_i}{1 + (\omega \tau_1)^2} + \frac{1 - \alpha_i}{1 + (\omega \tau_2)^2} \quad (3.14)$$

where i is the i th pixel in an image. α_i is the relative contribution of one of the lifetime components. In an AB plot, the semicircle represents all possible single-lifetime systems measured at a specific frequency, and a chord connecting two positions on the semicircle gives all possible values for a two component mixture with lifetimes given by the two points on the semicircle. A simulated example of an AB plot is shown in Fig. 3.6. One lifetime component τ_1 was set to be 2 ns, and the other component τ_2 was set to be 3 ns and 12 ns. When the system contains only one lifetime component, the results (the 2 ns, 3 ns, and 12 ns points) lie on the semicircle. When in a two lifetime system, by varying the contribution of the lifetime components, the results lie on the line connecting those two positions on the semicircle.

3.3 Summary

Based on the knowledge of fluorescence microscopy, the technique used in this thesis—fluorescence lifetime imaging microscopy—is then presented. Two types of FLIM, time-domain FLIM and frequency-domain FLIM and their (dis)advantages are compared. The theory behind FD-FLIM is presented.

Even though the market is dominated by TD-FLIM systems, in practice FD-FLIM has specific advantages over TD-FLIM and has also been widely used [73, 75–80]. For example, most of the TD-FLIM measurements are generally performed using confocal microscopes

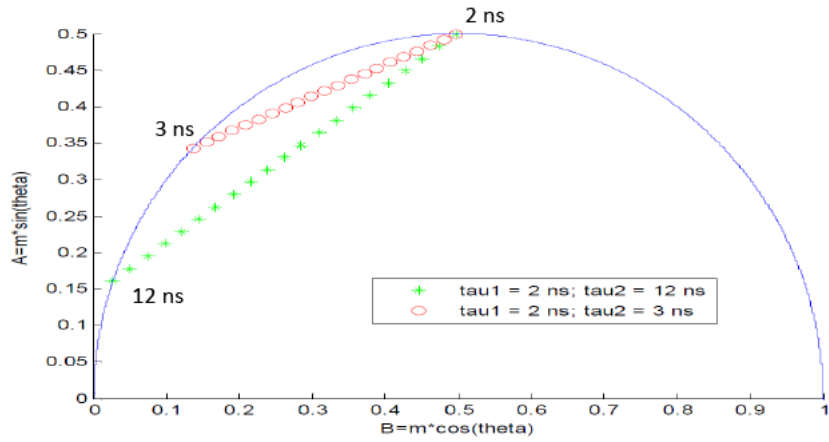


Figure 3.6: The illustration of an AB plot.

while FD-FLIM can also be done on widefield microscopes. For future applications in medical diagnostics, industrial inspection, and agriculture, this has obvious advantages. The use of the confocal microscope not only increases the cost of a TD-FLIM system, but also significantly increases the acquisition time for images. In standard FD-FLIM systems such as the one that we use as a reference system, image acquisition can be $100\times$ faster than a TD-FLIM system for an equivalent image size, typically 10 minutes for a TD-FLIM system and 5 seconds for an FD-FLIM system per lifetime image. The fast acquisition time makes it easier for FD-FLIM to monitor fast lifetime changes in cellular images. This, in turn, offers obvious advantages for future applications.

CHAPTER 4

Sensor and image intensifier

Abstract

Besides the microscope, another crucial part of FLIM is the image sensor. Charge-coupled devices (CCD) operation principles and different CCD sensor architectures are discussed in this chapter. The image intensifier, which is employed in the conventional frequency-domain FLIM, is introduced.

Keywords: Charge-coupled devices (CCD), image intensifier

4.1 Image sensors

An image sensor is a device which converts the optical signal to an electronic signal that is amplified, digitized and finally processed. Currently, there are two popular image sensor types: charge-coupled devices (CCD) [81] and complementary metal oxide semiconductor (CMOS) image sensors [82]. These two sensor types differ from each other in the way they process the acquired photoelectrons and the way they are manufactured. The CCD moves generated photo-electrons from pixel to pixel and converts them to a voltage at an output node while the CMOS converts the electrons to voltage inside each pixel, as shown in Fig. 4.1*. They have their own advantages and disadvantages, as shown in Table. 4.1 [82]. Extensive comparisons can be found in the literature such as [84–86]. The scientific CMOS camera (sCMOS) has an improvement on the dynamic range, full frame rate and noise aspect [87]. In this thesis, we focus on CCD based technology.

Table 4.1: Comparison of the advantages and disadvantages of CCD and CMOS technologies.

	CCD	CMOS
Sensitivity	High	Moderate
Image quality	Good	Moderate
Noise	Moderate	High
Dynamic range	High	Moderate
Power consumption	High	Moderate
Imaging speed	Moderate	Fast
Fill factor	High	Low
Blooming immunity	Bad	Good
Vertical Smear	Yes	No

4.1.1 CCD operation principle

The CCD was invented at Bell Telephone Laboratories in 1969 [86] by Willard S. Boyle and George E. Smith. For this they were awarded the Nobel Prize for physics in 2006. The CCD was originally invented to be a serial memory.

The CCD is composed of a series connection of Metal-Oxide-Semiconductor (MOS) capacitors. To capture an image, the light is projected onto the photoactive region of the capacitor arrays, which causes the capacitors to accumulate an electric charge proportional to the light intensity. The charge packages then can be transported from one capacitor

*Image source: [83].

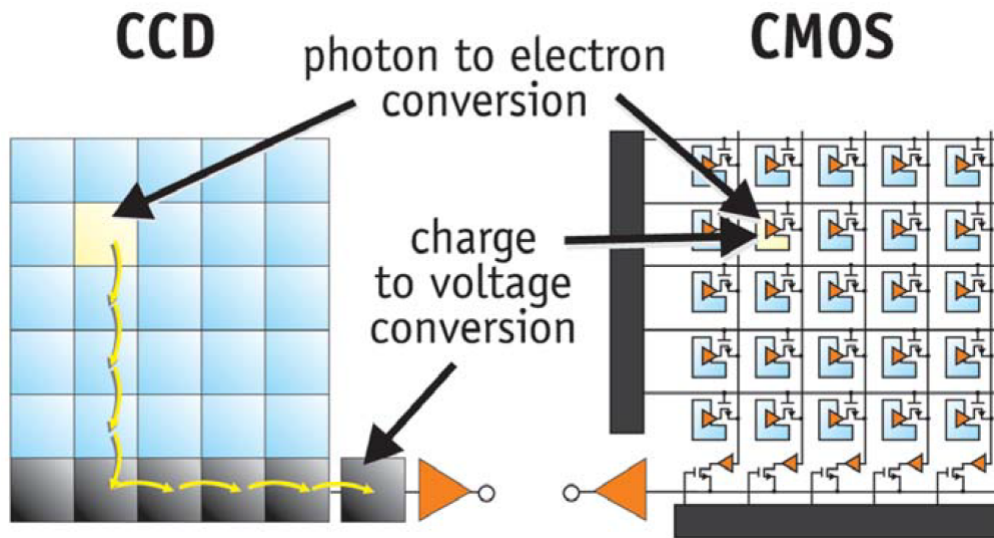


Figure 4.1: The difference between CCD and CMOS in image process level.

to another by manipulating the voltage applied on the gate electrodes on the top of MOS structures. The capacitors are arranged geometrically close to each other. The end of a chain of MOS capacitors is closed with an output node and an appropriate output amplifier, where the charges can be translated into a voltage and processed by other devices outside of the CCD image sensor [88]. Jerome Kristian and Morley Blouke used the concept of a network of buckets to describe the CCD principle, as shown in Fig. 4.2[†]. The brightness measurement in a CCD can be likened to using an array of buckets to measure the rainfall at different locations of a field. After the rain, the buckets in each row are moved across the field to conveyor belts, and are emptied into another bucket at the end of the conveyor, which carries the water into a metering bucket. The metering bucket carries out the conversion to voltage.

4.1.2 CCD architectures

Several different architectures can be implemented for CCD image sensors. Below we will discuss the most common architectures: full frame CCD, frame transfer CCD, and interline transfer CCD. Each architecture has its advantages and disadvantages; the choice of the architecture comes down to one's application purpose.

The illustration of the full frame CCD is shown in Fig. 4.3 (a). After a certain integration time, the photons are collected by the pixel elements and converted to the charges. All charge is shifted towards the serial readout register, one row at a time. The serial readout register then shifts each row to an output amplifier. The charges are then converted to a discrete number by an analog-to-digital converter (ADC). All the

[†]Imaging source: <http://www.astro.queensu.ca/~mhall/phy315/reduction.html>. 18 Sept 2012.

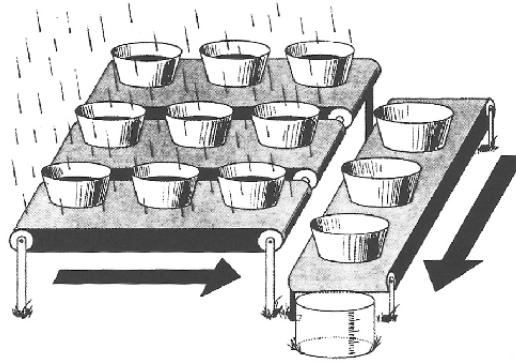


Figure 4.2: The bucket analogy used to describe CCD operation.

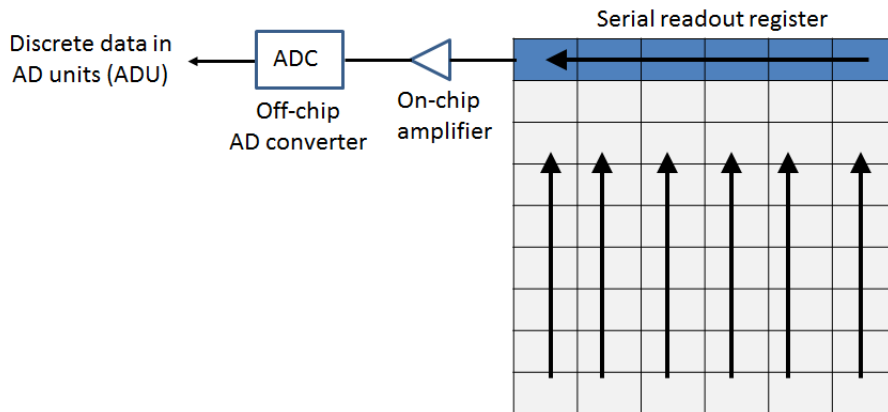


Figure 4.3: Device architecture of a full frame CCD.

charges in the serial readout register must be shifted out before the next row comes. The disadvantage of the full frame CCD is a mechanical shutter or a synchronized illumination scheme is needed to prevent smearing which is caused by light falling onto the sensor while the charges are being transferred to the readout register, a form of “motion-blur”. The advantage for the full frame CCD is that the whole pixel array is used to detect the photons and there is essentially no dead space between the adjacent pixels. This enables the full frame CCD to have a high sensitivity and a very high fill factor (the percentage of a pixel devoted to collecting photons), close to 100%.

The frame transfer CCD has an architecture similar to that of the full frame CCD, as shown in Fig. 4.4(a). In a frame transfer CCD, the sensor is divided into two identical areas. One area is sensitive to photons and used to capture the image. After the image is collected, the charges are rapidly transferred to the other half of the sensor, which is protected from the light and used as a memory array. Then the charge in the memory

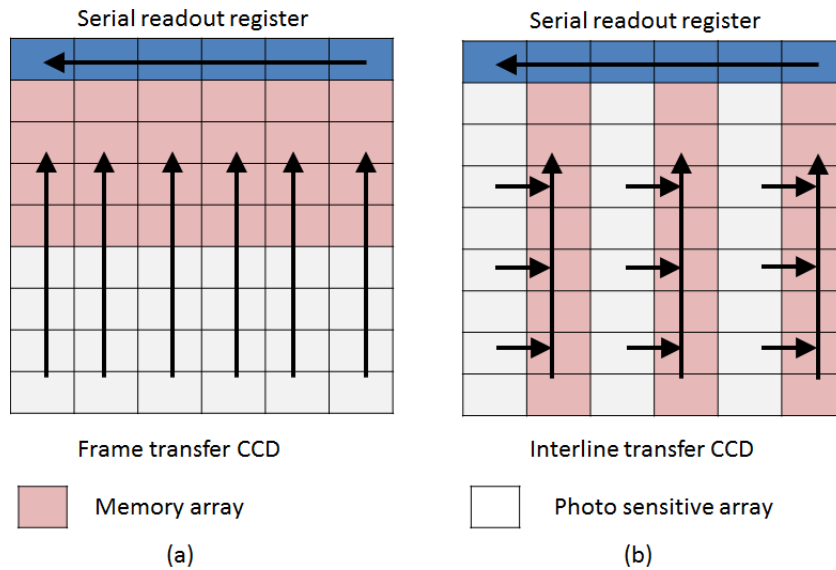


Figure 4.4: Device architectures of a frame transfer CCD (a) and an interline transfer CCD (b).

array can be slowly transferred to the serial readout register while the photo sensitive area collects new image data. The disadvantage of this architecture is that image smear is still possible. It is significantly better, however, when compared to the full frame CCD. Another downside of this architecture is that it needs twice the physical area compared to the full frame CCD in order to accommodate the memory array, thus increasing the cost of this architecture. The advantage is that the photo sensitive area is always collecting light which gives a high duty cycle (frame rate) and enables a continuous image readout. The sensitivity of the frame transfer CCD can be as good as that of the full frame CCD. The frame transfer CCD is normally employed in video cameras.

Another frequently employed architecture in video cameras is the interline transfer CCD. The interline transfer CCD extends the concept in the frame transfer CCD a step further. The memory array is located adjacent to the photo sensitive area, and every other column is shielded from the light to store the charge, as shown in Fig. 4.4 (b). In this way, the charge only needs to be shifted one pixel distance in the horizontal direction and the smear effect can be minimized. The charge will subsequently be shifted vertically towards a serial readout register. The interline transfer CCD, however, suffers from a low fill factor. This shortcoming can be improved by putting microlenses above the photo sensitive areas to increase the light collected into each sensor. The cost of this architecture is also high due to the low fill factor and the complex design.

4.2 Image intensifier

The conventional frequency domain fluorescence lifetime measurement requires an image intensifier, which serves two purposes. One is that the image intensifier is used to obtain a higher SNR by amplifying the incoming photons. The other function of the image intensifier in FLIM measurements is the demodulation process of the fluorescence signal.

4.2.1 The operating principle of the image intensifier

The image intensifier is normally used to boost the signal to noise ratio (SNR) in low light conditions or when the integral of the photon flux over the exposure time is very small. The image intensifier is placed in front of the CCD camera, as shown in Fig. 4.5. The image signal coming out of the microscope is projected on the photocathode of the image intensifier, which converts the detected photons to electrons. Each micro-channel acts as an electron multiplier: an electron entering a channel is forced through the channel by the electric field. When the electrons go through the micro-channel plate (MCP) inside of the image intensifier, they will hit the inner resistive surface of the channel, creating multiple secondary electrons. At the end of the MCP, the electrons hit a phosphorescent screen, which converts the electrons back to photons. The output signal of the image intensifier is an intensified copy of the input image signal that was projected on the photocathode. The image intensifier is connected to a CCD camera by fiber optics or relay lenses. Finally, the photons from the phosphorescent screen are converted to the photo electrons in the CCD sensors.

4.2.2 The demodulation principle of the image intensifier

In order to retrieve the phase delay and modulation change to calculate the lifetime, the fluorescence signal undergoes the demodulation process. The demodulation, in the conventional FD-FLIM is carried out on the image intensifier. A detailed illustration of the image intensifier is shown in Fig. 4.5. The gain of the image intensifier is modulated by applying a sinusoidal signal on the photocathode, as shown in Fig. 4.6.

The demodulation signal has the same frequency as the modulated light source as it is a homodyne system. The DC offset of this demodulation signal is chosen at the cutoff point of the image intensifier, which is the threshold voltage at which the electrons generated at the photocathode can be accelerated towards the MCP. In order to find the cutoff point, one can slowly increase the cathode DC voltage until the image begins to turn dark. Fig. 4.6 is a typical relationship between cathode DC voltage and the average image intensity of a region of interest. The camera used in this experiment is LI²CAM Intensified CCD camera (GenII with S25 photocathode) from Lambert Instruments (Roden, The Netherlands). The positive period of the cathode AC voltage will let none of the electrons through, while a negative period of the cathode AC voltage will “open” the intensifier. Different cathode DC bias around which the AC signal is superimposed, results in different

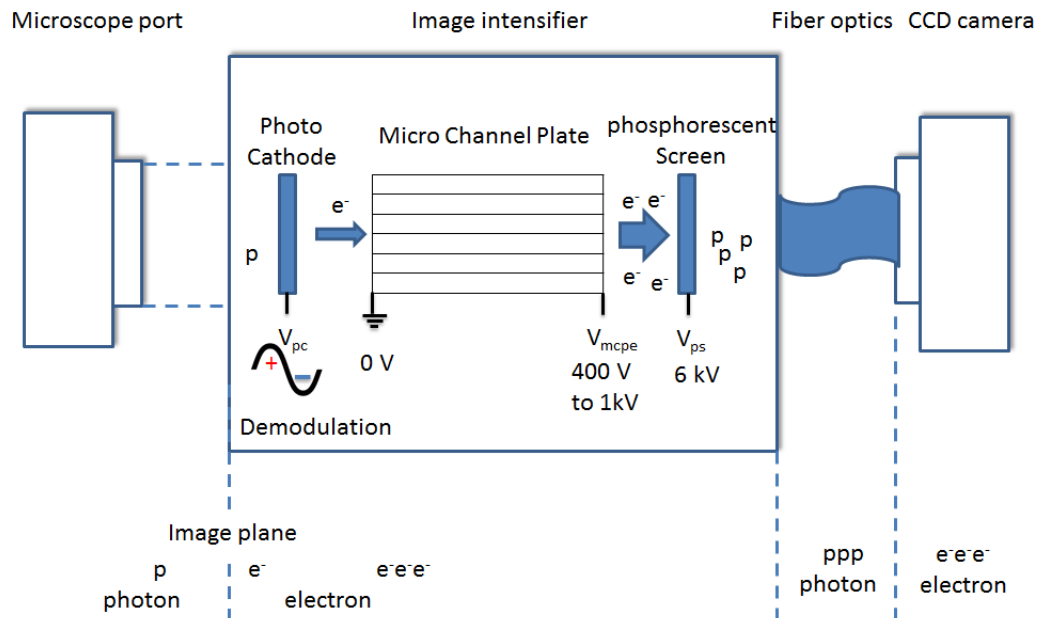


Figure 4.5: The image intensifier is normally placed in front of CCD camera.

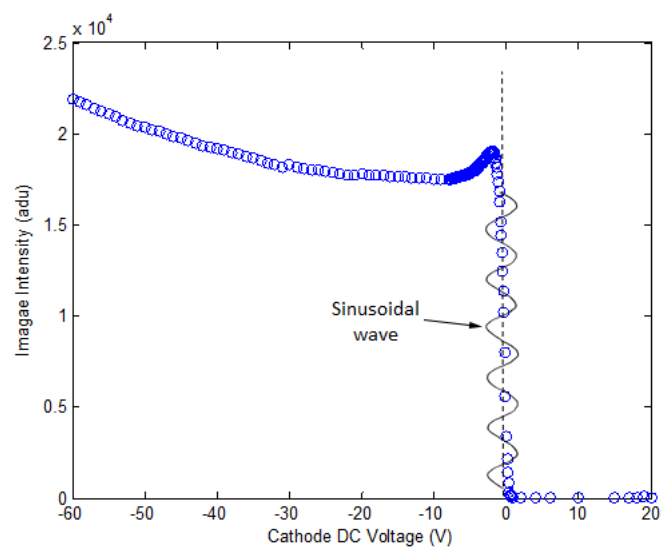


Figure 4.6: The average intensity of a region of interest at different cathode DC settings.

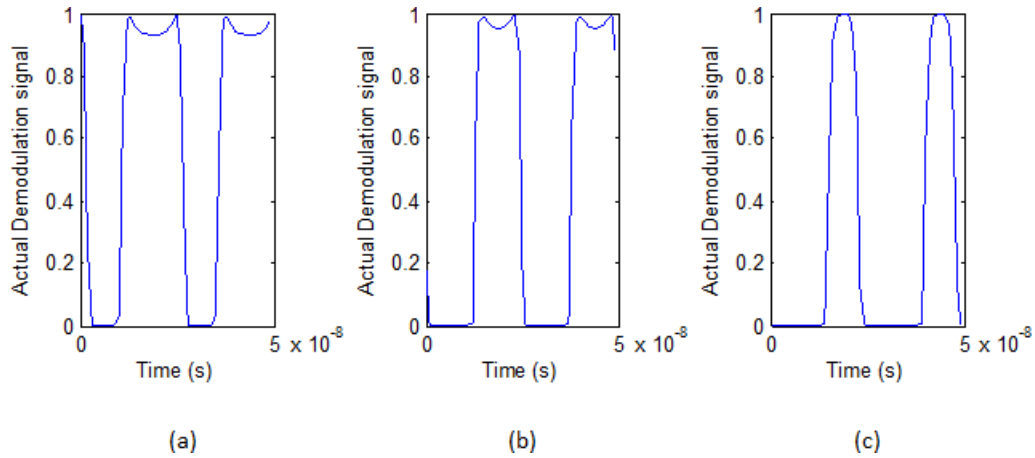


Figure 4.7: The same sinusoidal demodulation signal applied on different cathode DC settings. The DC biases are (a) -2 V, (b) 0 V, and (c) 2 V.

demodulation signals. An example is shown in Fig. 4.7. In Fig. 4.7, actual measured data from Fig. 4.6 is used to simulate demodulation signals when a pure sinusoidal AC signal is applied on the cathode DC bias. The sampling frequency is at 2 GHz. The voltage of cathode AC signal is set at 4 V, and the modulation frequency is 25 ns. The cathode DC bias is -2 V, 0 V, 2 V, respectively.

Before using an image intensifier based CCD camera for FD-FLIM measurements, one needs to calibrate the camera to the optimal setting since the cathode DC bias affects the precision of the lifetime measurements. On one hand, a higher DC bias results in a shorter (temporal) opening window. The opening time of the image intensifier is proportional to the cathode DC bias, as shown in Fig. 4.8. The simulation is done using the same parameters as the settings above in Fig. 4.7. A shorter opening time implies that fewer photons can be captured, which lowers the SNR. When the opening window gets shorter, however, the modulation depth of the gain gets higher (improves), as shown in Fig. 4.9. This higher modulation depth has a positive effect on the measurement precision. Thus the cathode DC bias, which leads to the smallest lifetime standard deviation should be used. To find this “sweet spot”, a green fluorescent plastic test slide which has a known lifetime of 2.8 ns was used [50]. There is insignificant bleaching in the test slide compared with fluorescent solutions, making it suitable for calibration. We keep the cathode AC the same while increasing the cathode DC bias step by step. Fig. 4.10 shows the measured lifetime precision (standard deviation) as a function of the cathode DC bias. In this case, when the cathode DC bias is smaller than 1.6 V, the lifetime precision is influenced more by the reduced SNR. When it is higher than 1.7 V, the higher modulation depth plays a dominant role. The best cathode DC bias is found at 1.6 V for lifetimes derived from the modulation depth change and 1.7 V for lifetimes derived from the phase change.

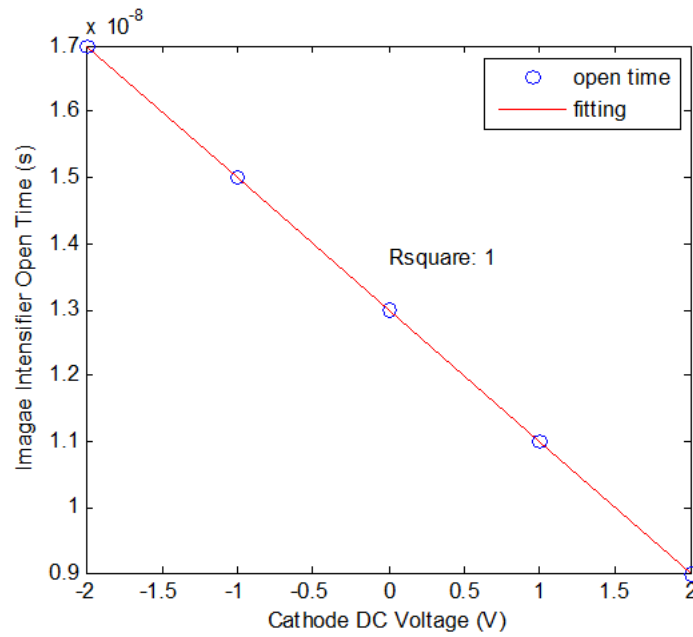


Figure 4.8: The simulated results of the relationship between cathode DC bias and the intensifier open time. The cathode DC bias set to (a) -2 V, (b) 0 V and (c) +2 V.

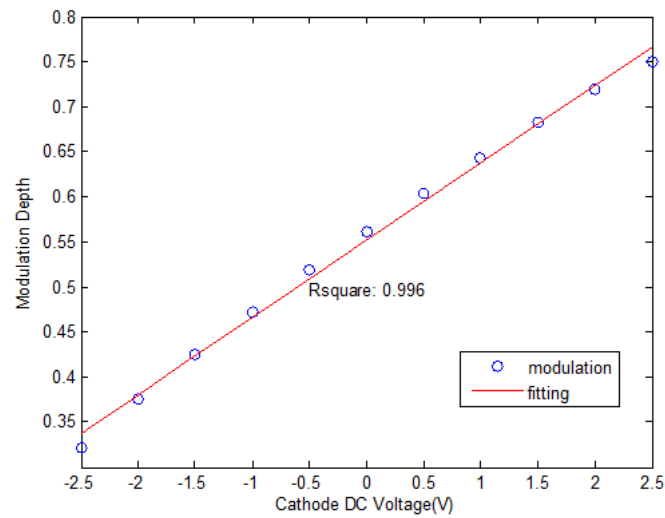


Figure 4.9: The simulated results of the relationship between cathode DC bias and the modulation depth of the signal.

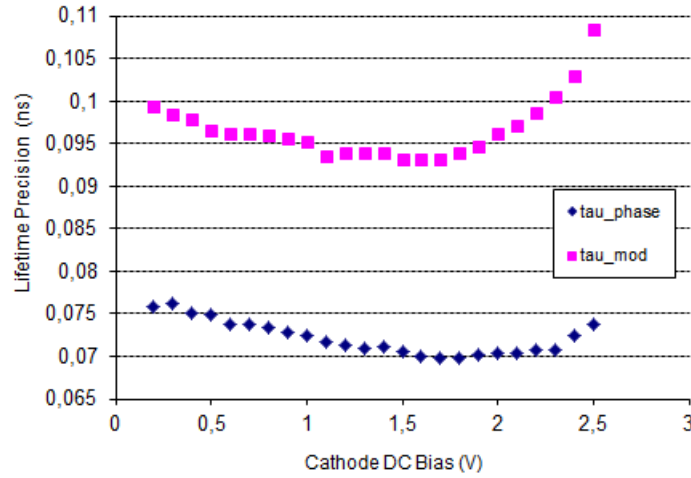


Figure 4.10: The lifetime precision influenced by the cathode DC bias.

4.2.3 The shortcomings of using image intensifier in FD-FLIM

To operate the image intensifier, high voltage up to several kilovolts is needed to be applied on the phosphorus screen. It requires elaborate electronics for the operation and is also relatively expensive. The spatial resolution will be compromised by the photocathode and the MCP. The image intensifier is vulnerable to over exposure. There will be geometric distortion due to the fiber coupling between the CCD and the intensifier, thus the fluorescence images might suffer from "chicken-wire" artifact, as shown in Fig. 4.11[‡]. Due to the operational principle, during half of the cycle there are no electrons travelling from the photo cathode to the MCP, which means half of the signal is lost during the demodulation. One major shortcoming of most image intensifier is irising at high frequencies [90]. Furthermore, the system is relatively costly, bulky, and vulnerable to overexposure. For these reasons, if a solid-state camera can replace the use of the image intensifier, it would be of great benefit.

4.3 Summary

This chapter introduces the concept of the CCD sensor and a comparison to a CMOS sensor. The CCD operational principle is discussed in this section. Three different types of CCD sensors are described: full frame CCD, frame transfer CCD and interline transfer CCD. The different versions of the developed MEM-FLIM sensors employed different CCD architectures described above.

This chapter also describes the architecture and the demodulation principle of the

[‡]The image source: [89]

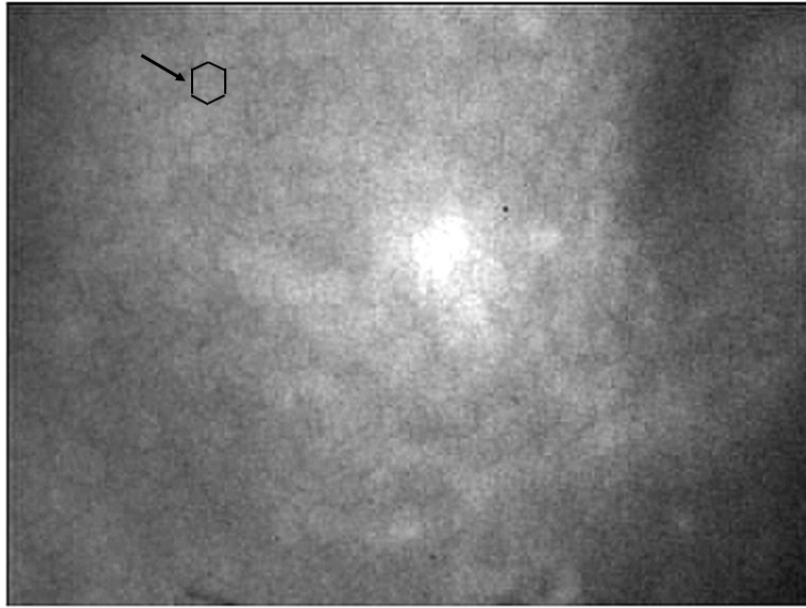


Figure 4.11: The chicken wire artifact introduced by image intensifier (the repeated patterns which the arrow points).

image intensifier. Image intensifiers are used in current FD-FLIM systems. The reason we pay attention to the image intensifier is that the developed MEM-FLIM camera is intended to eliminate the use of the image intensifier. Thus it is important to understand its function, strengths, and weaknesses in the current generation FD-FLIM systems.

CHAPTER 5

Photon Budget

Abstract

We have constructed a mathematical model to analyze the photon efficiency of frequency-domain fluorescence lifetime imaging microscopy (FLIM). The power of the light source needed for illumination in a FLIM system and the signal-to-noise ratio (SNR) of the detector have led us to a photon “budget”. These measures are relevant to many fluorescence microscope users and the results are not restricted to FLIM but applicable to widefield fluorescence microscopy in general. Limitations in photon numbers, however, are more of an issue with FLIM compared to other less quantitative types of imaging. By modeling a typical experimental configuration, examples are given for fluorophores whose absorption peaks span the visible spectrum from Fura-2 to Cy5. We have performed experiments to validate the assumptions and parameters used in our mathematical model. The influence of fluorophore concentration on the intensity of the fluorescence emission light and the Poisson distribution assumption of the detected fluorescence emission light have been validated. The experimental results agree well with the mathematical model. This photon budget is important in order to characterize the constraints involved in current fluorescent microscope systems that are used for lifetime as well as intensity measurements and to design and fabricate new systems.

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Keywords: fluorescence microscopy, fluorescence lifetime imaging microscopy (FLIM), photon efficiency, signal-to-noise ratio (SNR), light power

5.1 Introduction

Fluorescence microscopy has become an essential tool in biology and medicine. Whether fluorescence intensity, color, lifetime or any of the other properties that can be revealed (e.g. anisotropy) is being assessed, an understanding of the limitations induced by the observational instrumentation as well as the fluorescent process itself is necessary. We are developing a new generation of instrumentation for Fluorescence Lifetime Imaging Microscopy (FLIM) for reasons that will be described at the end of this manuscript. In this project we have found it essential to develop a model that links the number of excitation photons, the number of emission photons, and the signal-to-noise ratio (SNR) that would be present in a resulting digital image when the fluorescence data are acquired through a digital, microscope-based imaging system. Our resulting model, however, is equally applicable to widefield, fluorescence microscopy in general. But we begin with FLIM.

To quantify the performance of a frequency-domain lifetime imaging technique, photon efficiency, or “economy” as described by Esposito et al. in [79], has been studied by many researchers and an F-value has been used to describe a “normalized relative RMS noise” [71, 79, 91, 92]. Little attention, however, has been paid to the photon efficiency of the system. When Esposito et al. studied the relative throughput of a detection technique, the efficiency was considered to be 1 [79], which is normally not the case. In reality, many factors play a role in determining the system efficiency, such as the collection efficiency of an objective lens, the optical component light transmission or reflection efficiency, the fill factor and quantum efficiency of the camera, and so on [50, 93]. Clegg described the sensitivity of fluorescence measurement by listing some factors that require attention [6]. To better understand the constraints that are encountered in current and future microscope systems, a mathematical model has been developed to provide a quantitative photon budget analysis. In this photon budget, we focus on the choice of the light source for a FLIM system and the signal-to-noise ratio (SNR) that a camera should ultimately achieve. These subjects are relevant to many fluorescence microscope users and the results are not restricted to FLIM but applicable to widefield fluorescence microscopy in general. Limitations in photon numbers, however, are more of an issue with FLIM compared to other less quantitative types of imaging. Considerations associated with fluorescence resonance energy transfer (FRET), however, are excluded. We have also performed experiments to validate the assumptions used in the mathematical model.

5.2 Theory

A fluorescence system, consisting of an ensemble of molecules, can be considered for the most part as a linear time-invariant (LTI) system [94, 95]. It is linear because the weighted sum of two excitation signals will produce the weighted sum of two emission signals. Mathematically if $x_1(t) \rightarrow y_1(t)$ and $x_2(t) \rightarrow y_2(t)$, then $\alpha x_1(t) + \beta x_2(t) \rightarrow \alpha y_1(t) + \beta y_2(t)$, in which α and β are scaling factors. The system can be considered as time-invariant until photo-destruction of the fluorescent molecules occurs. This means that a delay in the excitation signal $x(t - t_0)$ will produce a corresponding delay in the

emission signal $y(t - t_0)$.

Since the fluorescence system is an LTI system with an impulse response characterized by the sum of one or more decaying exponentials, the fluorescence emission resulting from a sinusoidally-modulated excitation light source will also be modulated at the same frequency but with a phase shift and a decreased depth of modulation. The frequency-domain FLIM system uses a sinusoidally modulated light source and a detector modulated at the same frequency to calculate the lifetime. Note that the principle requirement is that the modulation and demodulation signals have the same Fourier harmonics. This allows, for example, the use of square-wave demodulation. A single lifetime can be calculated using Eq. (5.1) and/or Eq. (5.2) [96]:

$$\tau_\theta = \frac{1}{\omega} \tan(\theta) \quad (5.1)$$

$$\tau_m = \frac{1}{\omega} \sqrt{\frac{1}{m^2} - 1} \quad (5.2)$$

In these equations, θ is the phase change, ω is the angular frequency of the modulation, and m is the relative modulation depth of the emission signal compared to the excitation signal. These two derived lifetimes are only equal to the true fluorescence lifetime for mono-exponential homogeneous lifetime samples. Often, however, the sample being measured contains various quantities of differing lifetime species or species in a multiple of lifetime states. When this occurs, the lifetimes derived from the phase and from the modulation depth will no longer be equal. In order to determine the lifetimes in the presence of two or more lifetime components, the phase and modulation must be recorded at multiple frequencies, where the reciprocal of the frequencies are in general chosen so as to span the full lifetime range in the sample (typically 10-100 MHz for nanosecond fluorescence lifetimes). A minimum of N frequency measurements is required to discern N lifetime components [97].

In this section we will discuss the mathematical model required to determine (1) the power of the light source and (2) the resulting SNR at the detector.

5.2.1 Estimating the Power of the Light Source

A photon budget analysis describing the amount of light needed to excite a fluorescence sample is presented below. This analysis can be used to choose a suitable light source for a proposed FLIM system or for a (quantitative) fluorescence microscope system. Based on a hypothesized number of *emission* photons, the number of *excitation* photons is deduced by following the excitation path back to the light source, as shown in Fig. 5.1(a).

We assume that an $a \times a$ pixel camera is used, a square pixel size of $b \times b$ [meter²], and a total optical magnification of M . The numerical aperture of the objective lens is NA . The excitation wavelength is λ_{ex} [meter]. The volume of the voxel V that is associated with each imaged pixel at the specimen will approximately be:

$$V = (\Delta x)(\Delta y)(\Delta z) \approx \left(\frac{b}{B}\right)^2 \left(\frac{\lambda_{ex}}{2NA^2}\right) \quad [\text{m}^3] \quad (5.3)$$

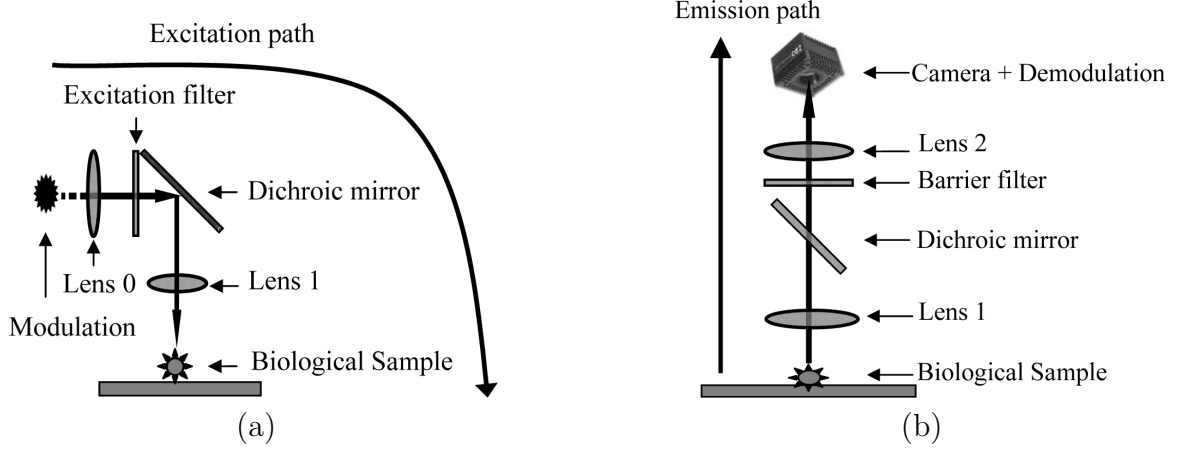


Figure 5.1: Illustration of the schematic for the photon budget analysis. (a) Excitation path that is used to calculate the power of the light source, and (b) emission path, which is used to deduce the SNR at the detector.

$$(\Delta z) \approx \frac{\lambda_{ex}}{2NA^2} \quad (5.4)$$

where Δz is the depth-of-field (DOF) [33]. Assuming that the fluorescent molecule concentration c [mol/m³] is given, then there will be m molecules per voxel:

$$m = cN_A \left(\frac{b}{B} \right)^2 \left(\frac{\lambda_{ex}}{2NA^2} \right) \quad [\text{molecules/voxel}] \quad (5.5)$$

in which, $N_A = 6.022 \times 10^{23} \text{ mol}^{-1}$ is Avogadro's constant. If c is expressed as a molar solution [mol/liter] then the proper conversion to [mol/m³] must be made.

Let us assume that each fluorescent molecule can emit n_{emit} photons before photo-destruction ends the fluorescence emission. One fluorescein molecule, for example, can emit 30000 to 40000 photons before it is permanently bleached [98]. The values for some other fluorescent molecules are given in Table 1. We can, therefore, expect to collect a maximum of $n_{emit} \cdot m$ photons per voxel. If the lifetime estimate requires the recording of r images, each of which takes T seconds, and the time interval between two recordings is identical and is T_0 seconds, then the average number of photons per recording will be:

$$\begin{aligned} n_{rec} &= \frac{n_{emit} T m}{rT + (r-1)T_0} \\ &= \frac{n_{emit} T}{rT + (r-1)T_0} \left(cN_A \left(\frac{b}{m} \right)^2 \left(\frac{\lambda_{ex}}{2NA^2} \right) \right) \quad [\text{photons/recording/voxel}] \end{aligned} \quad (5.6)$$

For the conventional application, widefield fluorescence microscopy, we set $r = 1$. We assume, but do not recommend, that the excitation light is left on during the $(r-1)T_0$ inter-recording intervals. If this is not the case and the excitation light is switched off, then we can set $T_0 = 0$ in Eq. (5.6). Excitation photons that enter a volume containing

fluorophores are either absorbed within the volume or pass through it. It is not important to know by what mechanism they leave the volume, e.g. direct transmission or scattering. What is important is that they are not absorbed. We refer to the number of excitation photons entering the volume as n_0 and the number of emission photons exiting the volume as n_1 . Not every absorbed photon produces an emission photon and the ratio emitted to absorbed is the quantum yield Φ , with typical values being $0.5 < \Phi < 1$. An ideal fluorophore would have a quantum yield close to unity.

Emission photons either leave the volume or they remain in the volume through re-absorption. Using Eq. (5.5) and Eq. (5.6), the relation between a) the net number of photons that are emitted from a volume and thus could be recorded in an image and b) the photons that are (re)absorbed and thus do not leave the volume is given by:

$$\begin{aligned} \Phi n_{\text{absorb}} &= \Phi(n_0 - n_1) = n_{\text{rec}} \quad [\text{photons/recording}] \Rightarrow \\ n_{\text{absorb}} &= \frac{n_{\text{emit}} T m}{[rT + (r-1)T_0]\Phi} \quad [\text{absorbed photons/recording}] \end{aligned} \quad (5.7)$$

According to the Beer-Lambert law, we can relate the number of photons entering the volume n_0 to the number of photons leaving the volume by:

$$n_1 = n_0 \times 10^{-A} \quad (5.8)$$

where A is the absorption coefficient. Using Eq. (5.4) and Eq. (5.5), the absorption coefficient A for one voxel path length Δz is:

$$A = \varepsilon(\lambda_{ex}) c \Delta z = \varepsilon(\lambda_{ex}) \left(\frac{m}{N_A \left(\frac{b}{M}\right)^2 \left(\frac{\lambda_{ex}}{2NA^2}\right)} \right) \left(\frac{\lambda_{ex}}{2NA^2} \right) = \frac{\varepsilon(\lambda_{ex}) m M^2}{N_A b^2} \quad (5.9)$$

where $\varepsilon(\lambda_{ex})$ [m^2/mol] is the molar extinction coefficient of the fluorescent molecule. The SI units for $\varepsilon(\lambda_{ex})$ are m^2/mol , but in practice, they are usually taken as $\text{M}^{-1}\text{cm}^{-1}$. The value of $\varepsilon(\lambda_{ex})$ depends on the excitation wavelength.

Our choice of a “volume” needs some elaboration. First, as we are using epi-illumination, a single microscope objective for the excitation path as well as the emission path, we assume that the volume of the sample that is being excited is the same as the volume that is observed for fluorescence. The approximate dimensions of this volume are the area in the lateral plane of one pixel $(b/M)^2$ and the value of Δz given in Eq. (5.4) in the axial path. The amount of intensity that is to be found in this volume compared to the total volume that is illuminated and examined is about 70%. This value follows from direct application of the theory described in [33][Section 8.8.3, Eq.39].

Solving for the number of excitation photons needed to produce the number of absorbed photons per recording (r) gives:

$$n_0 = \left(\frac{1}{1 - 10^{-A}} \right) n_{\text{absorb}} = \frac{T m n_{\text{emit}}}{[rT + (r-1)T_0]\Phi \left(1 - 10^{-\frac{\varepsilon(\lambda_{ex}) m M^2}{N_A b^2}} \right)} \quad [\text{photons/recording}] \quad (5.10)$$

We use n_0 as the maximum value per voxel. If more excitation photons are used than this, then the molecules will bleach before the necessary number of recordings has been made.

As shown in Fig. 5.1, the reflection efficiency of the dichroic mirror R_D , the transmission efficiency of the excitation filter τ_{EF} , and the transmission efficiency of the lenses in the excitation path τ_{lens01} should also be considered. R_D , τ_{EF} , τ_{lens01} are all wavelength dependent, but for notational simplicity we will forego using an explicit notation such as $R_D(\lambda)$. The number of photons from the light source needed to produce n_0 excitation photons will, therefore, be:

$$n_{0source} = \left(\frac{n_0}{R_D \tau_{EF} \tau_{lens01}} \right) = \frac{T m n_{emit}}{[rT + (r-1)T_0] R_D \tau_{EF} \tau_{lens01} \Phi \left(1 - 10^{-\frac{\varepsilon(\lambda_{ex}) m M^2}{N_A b^2}} \right)} \quad (5.11)$$

[photons/recording/pixel]

The number of excitation photons, $n(\lambda_{ex})$, per second required for illumination of the entire field of view (as opposed to just one pixel) will be:

$$n_i(\lambda_{ex}) = \left(\frac{a^2 n_{0source}}{T} \right) = \frac{a^2 m n_{emit}}{[rT + (r-1)T_0] R_D \tau_{EF} \tau_{lens01} \Phi \left(1 - 10^{-\frac{\varepsilon(\lambda_{ex}) m M^2}{N_A b^2}} \right)} \quad (5.12)$$

[photons/s/image]

If the energy from the light source is E_{ex} [J/photon], then the power W of the light source required for excitation of the entire field of view is:

$$W = n_i E_{ex} = \frac{a^2 m n_{emit} E_{ex}}{[rT + (r-1)T_0] R_D \tau_{EF} \tau_{lens01} \Phi \left(1 - 10^{-\frac{\varepsilon(\lambda_{ex}) m M^2}{N_A b^2}} \right)} \quad [\text{Watts}] \quad (5.13)$$

5.2.2 Estimating the SNR at the detector

We can identify four possible noise sources for digitized fluorescence images: photon noise due to the fundamental (quantum) physics of photon production (P), dark current noise due to the production of photoelectrons through thermal vibrations (D), readout noise due to the analog and digital electronics (E), and quantization noise due to the process of converting an analog intensity value into a quantized gray level (Q). These noise sources are mutually independent and this means that the total noise variance σ_T^2 is the sum of each of the noise variances: $\sigma_T^2 = \sigma_P^2 + \sigma_D^2 + \sigma_E^2 + \sigma_Q^2$. Through cooling, as with a Peltier element, and short integration times-in our case this is about 200 ms-the dark current contribution, σ_D^2 , can be neglected, that is $\sigma_D^2 \approx 0$. Through proper electronics design the readout contribution, σ_E^2 , can be neglected. The ADC readout noise, for example, is dependent on the ADC readout frequency-in our system it is 11 MHz-and is, thereby, reduced to manageable levels, that is $\sigma_E^2 \approx 0$.

This leaves the contributions from photon noise and quantization noise, $\sigma_T^2 = \sigma_P^2 + \sigma_Q^2$. We begin with photon noise and denote the signal-to-noise ratio for photon noise as simply SNR.

The SNR at the detector is calculated by analyzing the photon loss in the *emission* path, as shown in Fig. 5.1(b). We assume that the total number of photons that a single fluorescent molecule can emit before photo-destruction occurs is n_{emit} . Allowing r phase recordings, each of which takes T seconds, and the time interval between two recordings as T_0 seconds, n_{epr} photons are emitted on average and thus can be used per recording.

$$n_{epr} = \frac{n_{emit}T}{rT + (r-1)T_0} \quad [\text{usable photons/recording}] \quad (5.14)$$

But not all of these photons will be collected by the objective lens. The numerical aperture (NA) describes the light collection ability of a lens and is given by:

$$NA = n \sin \theta \quad (5.15)$$

in which θ is the acceptance angle of the lens, and n the index of refraction of the immersion medium of the lens. The number of photons, which have the chance to reach and be captured by the lens (n_{lens}), is dependent upon θ .

Figure 5.2(a) illustrates the isotropic emission of fluorescence photons and the fraction captured by the objective lens. The number of photons that can be captured by the lens n_{lens} within an angle θ is:

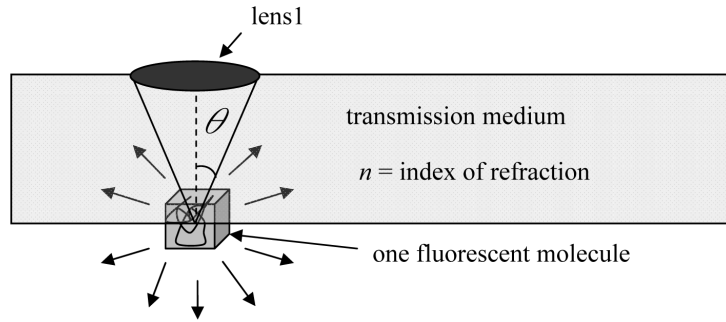
$$n_{lens} = n_{epr}(1 - \cos \theta)/2 \quad (5.16)$$

The factor of $1/2$ in the above equation comes from the fact that only half of the isotropically emitted photons travel towards the lens. The photon capture efficiency γ of the lens is described in Eq. (5.17) and is the photon number that the lens can capture divided by the total number of photons that the fluorescent molecules emit. Fig. 5.2(b) shows the photon capture efficiencies for different immersion media such as air ($n = 1.0$), water ($n = 1.33$) and oil ($n = 1.51$). Typical values of different lenses are marked as dots in the figure.

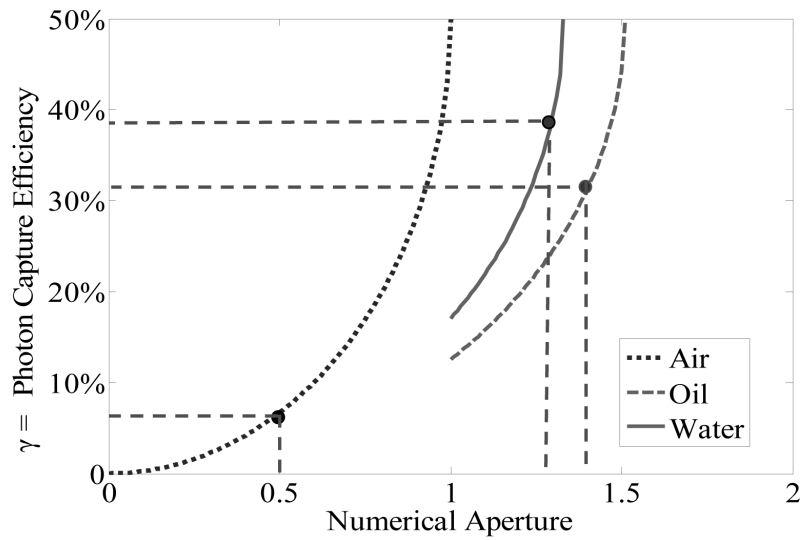
$$\gamma = \frac{n_{lens}}{n_{epr}} = \frac{1 - \cos \theta}{2} = \frac{1 - \sqrt{1 - \sin^2 \theta}}{2} = \left(1 - \sqrt{1 - (NA/n)^2}\right)/2 \quad (5.17)$$

The transmission efficiencies of the objective lens, the dichroic mirror, the barrier filter, and the second lens are denoted τ_{lens1} , τ_D , τ_B and τ_{lens2} , respectively. The transmission coefficient of the camera window is τ_w , the fill factor is F , and the quantum efficiency is η . The parameters τ_{lens1} , τ_D , τ_B , τ_{lens2} , τ_w and η are emission wavelength dependent but again, we suppress the functional dependency on λ in favor of notational simplicity. The ratio of the CCD area to the excitation spot area is κ . Then the number n_e of photoelectrons detected by the camera will be:

$$n_e(\lambda) = \underbrace{(\tau_{lens1}\tau_D\tau_B\tau_{lens2}\tau_w\eta)}_{\text{wavelength dependent}} \kappa F n_{epr}(\lambda) \left(1 - \sqrt{1 - (NA/n)^2}\right)/2 \quad (5.18)$$



(a)



(b)

Figure 5.2: Photon-capture efficiency of the objective lens. (a) Illustration of the directions of photons emitted by a fluorescent molecule and that portion captured by the objective lens. (b) The fraction of photons captured by various lenses compared to the photons emitted by one fluorescent molecule. If the immersion medium is air $n = 1$, $0 \leq NA \leq 1$; if it is water, $n = 1.33$, $NA > 1$; and if it is immersion oil, $n = 1.51$, $NA > 1$. Values for different objective lenses (Nikon, Fluor Ph2DL, 10x, NA 0.5; Nikon, Plan Fluor 100x, NA 1.3; Zeiss, Plan, 63x, NA 1.4) are marked as dots in the figure.

We assume in this manuscript, for the sake of simplicity, that the terms in Eq. (5.19) that vary over the emission wavelengths of interest, ($\lambda_1 \leq \lambda \leq \lambda_2$), can be replaced by zeroth-order (constant) terms. We are essentially appealing to the Mean Value Theorem of calculus. This allows us to go from line two to lines three and four in Eq. (5.19). The total number of photoelectrons would then be given by:

$$\begin{aligned}
 n_e &= \int_0^\infty n_e(\lambda) d\lambda \\
 &= \left(\int_0^\infty \tau_{lens1} \tau_D \tau_B \tau_{lens2} \tau_w \eta n_{epr}(\lambda) d\lambda \right) \left(\kappa F \left(1 - \sqrt{1 - (NA/n)^2} \right) / 2 \right) \\
 &= (\tau_{lens1} \tau_D \tau_B \tau_{lens2} \tau_w \eta) \underbrace{\left(\int_{\lambda_1}^{\lambda_2} n_{epr}(\lambda) d\lambda \right)}_{n_{epr}} \left(\kappa F \left(1 - \sqrt{1 - (NA/n)^2} \right) / 2 \right) \quad (5.19) \\
 &= (\tau_{lens1} \tau_D \tau_B \tau_{lens2} \tau_w \eta) \left(\kappa F \left(1 - \sqrt{1 - (NA/n)^2} \right) / 2 \right) n_{epr}
 \end{aligned}$$

Two remarks are appropriate. First, as described by Roper Scientific and Andor Technology, the quantum efficiency of a standard, front-illuminated CCD chip over the FWHM emission wavelength range of GFP ($496 \text{ nm} \leq \lambda \leq 524 \text{ nm}$), can be extremely well approximated by $\eta = 24\%$ over this entire interval. Other “special” CCD chips such as those used by the Santa Barbara Instrumentation Group can be well approximated by $\eta = 71\%$ over this interval. Thus, the value of η may vary from chip-to-chip but the use of a constant value over the wavelength interval for a given chip is justified.

Second, and perhaps more importantly, the term n_{epr} in Eq. (5.19) represents the number of emission photons within the range ($\lambda_1 \leq \lambda \leq \lambda_2$), a number that is dependent upon the emission spectrum of the fluorescent molecule and the barrier and dichroic filters. For our GFP example, where $\lambda_1 = 502 \text{ nm}$ and $\lambda_2 = 538 \text{ nm}$ -see filter and experiment descriptions below-approximately 61% of the emitted photons are within this wavelength range.

Assuming the number of photons recorded during a fixed measuring period is random and described by a Poisson distribution [99], the SNR is defined and given by [100]:

$$\begin{aligned}
 SNR &= \frac{\text{average}}{\text{std.deviation}} = \frac{\mu}{\sigma} \\
 &= \frac{\langle n_e \rangle}{\sqrt{\langle n_e \rangle}} = \sqrt{\langle n_e \rangle} \\
 &= \left(\frac{(\tau_{lens1} \tau_D \tau_B \tau_{lens2} \tau_w \eta F \kappa) T n_{emit} \left(1 - \sqrt{1 - (NA/n)^2} \right)}{2[rT + (r-1)T_0]} \right)^{1/2} \quad (5.20)
 \end{aligned}$$

When expressed in the logarithmic units commonly used for electro-optics this becomes $SNR = 20 \log_{10}(\mu/\sigma) = 10 \log_{10}(n_e) \text{ dB}$. A more rigorous calculation of the SNR would

involve taking the wavelength dependency of the various terms in Eq. (5.20) into consideration, that is, performing an integration over the relevant wavelengths. The terms τ_D , τ_B , and n_{emit} have the most significant variations as a function of wavelength but for this analysis, as explained above, we use the simplest approximation of their being constant.

The average of n_e ($\langle n_e \rangle$) is calculated over the CCD pixels. With an electronic gain g [ADU/e], the conversion of photoelectrons to A/D converter units N [ADU] is described by $N = g \cdot n_e$. The average and standard deviation of N can be easily obtained: $\langle N \rangle = g \langle n_e \rangle$, $\sigma(N) = g(\langle n_e \rangle)^{1/2}$. Thus the SNR after conversion is the same as that before conversion, which indicates that the ADC conversion factor does not change the fundamental SNR, but only the observed grey level dynamic range.

There is a slight amount of quantization noise introduced by the ADC but that noise is, in general, negligible when compared to photon noise from fluorescence. The reasoning is as follows. Without loss of generality, the signal can be normalized to the interval $0 \leq \text{signal} \leq 1$. This is quantized into 2^b uniformly spaced intervals each of width $q = 2^{-b}$ where b is the number of bits. Replacing the analog value with the digitized value is equivalent to adding uniformly-distributed noise to the original value where the noise distribution has a mean of 0 and a variance of $\sigma_Q^2 = q^2/12$. The SNR_Q for this signal is defined as $SNR_Q = (\max \text{ signal})/\sigma_Q = \sqrt{12}/q = \sqrt{12} \cdot 2^b$. Rewriting this in logarithmic (dB) form gives $SNR_Q = 6b + 11$ dB [100]. For a 10-bit ADC, the $SNR_Q = 71$ dB. This is much higher than the typical SNR per pixel and can thus be ignored leaving the photon noise as the limiting factor.

5.3 Materials and methods

5.3.1 System configuration

Our baseline FLIM system includes an Olympus inverted microscope system IX-71 (Olympus), a LIFA system (Lambert Instruments, Roden, The Netherlands), a LI²CAM Intensified CCD camera (Lambert Instruments, Roden, The Netherlands) and a Dell computer installed with the Windows XP operating system.

A Zeiss objective with a magnification of $20\times$ and a numerical aperture of 0.5 has been used. The lateral resolution associated with the GFP emission wavelength is $\lambda_{em}/(2NA) = 509$ nm and the axial resolution is $\lambda_{em}/(2NA^2) = 1018$ nm. The dependence of the SNR on the NA is given explicitly in Eq. (5.20). Our LIFA system uses LED excitation with an emission peak at $\lambda = 469$ nm (Lambert Instruments, Roden, The Netherlands) in combination with a 472 ± 15 nm single-band excitation filter (FF01-472/30-25, Rochester, U.S.A). A 495 nm LP dichroic mirror (Semrock FF495-Di02-25 $\times$ 36, Rochester, U.S.A) is used in the fluorescence filter cube. The fluorescence is observed through a 520 ± 18 nm single-band emission filter (Semrock FF01-520/35-25, Rochester, U.S.A).

The LED DC current setting, via LI-FLIM software version 1.2.6 developed by Lambert Instruments, controls the intensity of the LED. Light power is measured using a laser power meter Ophir Model No. PD-300-SH (Jerusalem, Israel).

5.3.2 Materials

To determine the effect of the fluorophore concentration on the emission light, Rhodamine 6G (Sigma Aldrich 83697) was diluted in deionized water to different concentrations: 10, 50, 100, 250, 500, 1000, and 2500 μM . Rhodamine was held between a single well pattern microscope slide (Fisher Scientific 361401) and a cover slip (Menzel-Glaser 18 mm $\times$ 18 mm). For the focus of the Rhodamine 6G solution, we 1) focus on the edge of the solution, then 2) move the sample so that the middle of the solution sits above the objective pupil, and then 3) move the focus point into the solution by 50 μM using the indexed focusing knob.

A green fluorescence plastic test slide (Lambert Instruments) is used for validating the Poisson distribution assumption of the detected emission light, in order to avoid photobleaching either a biological sample or a fluorophore solution.

5.3.3 Determining the power of the light source

Let us look at some typical values and take fluorescein as an example. We have chosen fluorescein because, as shown in Table 5.1, it is almost a worst-case example. It provides a relatively small number of emission photons before photo-destruction. The total number of photons that a single fluorescein molecule can emit before photo-destruction occurs is $n_{emit} \approx 30000$ [98]. Fluorescein has a molar extinction coefficient of $\epsilon(\lambda_{ex}) = 59,668 \text{ M}^{-1}\text{cm}^{-1}$ at 488 nm excitation light [101]. The quantum yield is $\Phi = 0.9$ in basic solution [102]. We assume a molecular concentration of $c = 2 \text{ } \mu\text{M}$. Further, we assume that an $a \times a = 512 \times 512$ pixel camera is used with a square pixel size of $b = 25 \text{ } \mu\text{M}$ and a total optical magnification of $M = 100$. We assume that at the wavelengths of interest the reflection efficiency of the dichroic mirror is $R_D = 95\%$, the transmission efficiency of the excitation filter is $\tau_{EF} = 95\%$ [103], and the transmission efficiency of the lenses in the excitation path are $\tau_{lens01} = 96\% \times 96\% \approx 92\%$. The numerical aperture $NA = 1.3$. A monochromatic 488 nm laser source is assumed for the excitation source. Allowing $r = 12$ different phase recordings, one recording takes $T = 200$ milliseconds, and the time interval between two measurements is $T_0 = 0 \text{ s}$.

If we were to consider fluorescent molecules other than fluorescein, then the relevant fluorophore parameters needed to calculate the light power or SNR would be those given in Table 5.1. The equations and their derivations associated with some of the values in Table 5.1 and the following will be discussed in section 5.4.1. Table 5.1 should be used with care as it presents the optical power required if one wants to extract every possible emission photon from a molecule. If a fewer number of photons is required to achieve a desired goal-measurement of fluorescence lifetime with a certain precision, for example-then a lower power light source could suffice.

5.3.4 Determining the SNR at the detector

Using Eqs. (5.14)-(5.20), the number of photoelectrons that can be ultimately detected in FLIM can be calculated. We assume, for example, a $NA = 1.3$ objective lens with

Table 5.1: The light power needed to produce the maximum number of emission photons from a single fluorescent molecule in 0.2 s. The values have been calculated for nine different fluorophores whose absorption peaks span the visible spectrum. The calculations are based upon the data in this table and Eq. (5.13). As the maximum number of emission photons is a statistical average over an ensemble of identical molecules, all values are averages. (§This value is estimated from [98].)

Fluorophore	“Maximum” Number of Photons per Molecule	Molar Extinction Coefficient [M ⁻¹ cm ⁻¹]	λ_{ex} Peak [nm]	λ_{em} Peak [nm]	Quantum Yield Φ	Light Source Power [mW]	References
Fura-2	250	2.19×10^4	380	512	0.50	0.2	[104, 105]
GFP	4×10^5	4.4×10^4	446	509	0.77	94	[106–108]
Fluorescein	3×10^4	5.97×10^4	494	525	0.90	5	[98, 101, 102]
EYFP	10^5 (§)	8.34×10^4	514	527	0.60	14	[98, 108, 109]
Rhodamine 6G	1.1×10^6	1.16×10^5	530	555	0.95	67	[110–112]
Alexa546	3.5×10^6	1.12×10^5	561	572	0.79	250	[108, 110, 113]
Cy3	4.8×10^6	1.25×10^5	550	570	0.15	1.7×10^2	[108, 110, 114]
TMR	1.2×10^6	1×10^5	550	573	0.71	109	[110, 115, 116]
Cy5	9.9×10^4	2.5×10^5	650	666	0.23	p	[108, 117, 118]

oil as the medium for which the index of refraction is $n = 1.51$. Continuing with the fluorescein model, the quantum efficiency of the camera system, which depends upon the wavelength, is about $\eta(\lambda \approx 525\text{nm}) \approx 30\%$. We assume the camera fill factor $F = 40\%$, the transmission efficiency of the dichroic mirror is $\tau_D = 90\%$, and that of the barrier filter is $\tau_B = 95\%$ [103]. We assume the transmission of both lenses and the camera window are $\tau_{lens1} = \tau_{lens2} = \tau_w = 96\%$ and that the total number of photons that a single fluorescent molecule can emit is $n_{emit} \approx 30,000$. We assume the total phase recording number $r = 12$, and there is no time interval between two recordings $T_0 = 0$. If an $a \times a$ pixel camera is used and the diameter of the excitation circular spot is the same as the diagonal of the CCD chip, $\kappa = 2/\pi$. In reality the diameter of the excitation spot will be larger than the diagonal of the CCD chip, so we make an approximation that $\kappa = 1/2$.

To calculate the SNR for other fluorophores the critical parameters that may need to be changed are the total number of photons that a single molecule can emit before photo-destruction occurs and the quantum efficiency of the camera system at a possibly different emission wavelength. Such values are shown in Table 5.2. The derivation will be discussed later in section 5.4.2.

5.3.5 Assumptions and parameter validation

We have performed a series of experiments to validate the parameter values and assumptions used in our photon efficiency model. Considering the transmission efficiency of the optical components (filters and lens) as a single constant factor in the mathematical model is reasonable but will be tested. The influence of dye concentration on the intensity of the fluorescence emission light and the Poisson distribution assumption of the fluorescence emission light must certainly be validated. Standard Köhler illumination is used in these experiments.

5.3.5.1 Transmission efficiency of the optical components

In the mathematical model, the transmission efficiency of the optical components is treated as a constant parameter. To validate this, we measure the light at the source and the light exiting from the objective lens using the laser power meter. The LED DC current was varied from 10 mA to 150 mA. The power of the light coming out of the objective lens was then divided by the power of the light at the source to determine the transmission efficiency of the optical component chains.

5.3.5.2 Influence of concentration on the detected fluorescence emission intensity

In estimating the required power of the light source, we assume that the Beer-Lambert law describes the relation between excitation photon number and emission photon number as shown in Eq. (5.8). To express Eq. (5.8) in another way, the fluorescence emission photon number n_{rec} equals the product of the excitation photon number n_0 and an absorption

Table 5.2: Using Eq. (5.20) the SNR at the detector is calculated for the nine different fluorophores from Table 5.1. The SNR is evaluated for a single molecule and at a concentration of $c = 2 \mu\text{M}$ for a single pixel and for an entire $512 \times 512 \text{ image}$. As in Table 5.1, all values are averages. (§This value is estimated from [98].)

Material	“Maximum” Number of Photons per Molecule	λ_{em} Peak [nm]	Camera Quantum Efficiency at λ_{em}	SNR per Molecule	SNR for a Pixel $c = 2 \mu\text{M}$	SNR for an Image $c = 2 \mu\text{M}$	References
Fura-2	250	512	0.3	0.5 : 1 (-6 dB)	1.4 : 1 (3 dB)	7×10^2 : 1 (57 dB)	[104, 119, 120]
GFP	4×10^5	509	0.3	19 : 1 (26 dB)	61 : 1 (36 dB)	3×10^4 : 1 (90 dB)	[106, 119, 120]
Fluorescein	3×10^4	525	0.3	5 : 1 (14 dB)	18 : 1 (25 dB)	9×10^3 : 1 (79 dB)	[98, 119, 120]
EYFP	10^5 (§)	527	0.3	10 : 1 (20 dB)	32 : 1 (30 dB)	2×10^4 : 1 (84 dB)	[98, 119, 120]
Rhodamine 6G	1.1×10^6	555	0.35	35 : 1 (31 dB)	120 : 1 (42 dB)	6×10^4 : 1 (96 dB)	[110, 119, 120]
Alexa546	3.5×10^6	572	0.38	64 : 1 (36 dB)	222 : 1 (47 dB)	1×10^5 : 1 (101 dB)	[110, 119, 120]
Cy3	4.8×10^6	570	0.38	75 : 1 (38 dB)	260 : 1 (48 dB)	1×10^5 : 1 (102 dB)	[110, 119, 120]
TMIR	1.2×10^6	573	0.38	38 : 1 (31 dB)	130 : 1 (42 dB)	7×10^4 : 1 (96 dB)	[110, 119, 120]
Cy5	9.9×10^4	666	0.5	12 : 1 (22 dB)	46 : 1 (33 dB)	2×10^4 : 1 (88 dB)	[117, 119, 120]

factor $(1 - 10^{-c \cdot \epsilon \cdot \Delta z})$, as shown in Eq. (5.21).

$$n_{rec} = \Phi n_{absorb} = \Phi n_0 (1 - 10^{-c \cdot \epsilon \cdot \Delta z}) = B(1 - 10^{cD}) \quad (5.21)$$

B is proportional to the power of the excitation light, which is controlled by the LED DC current setting; D is the product of the molar extinction coefficient and the absorption path length, $D = \epsilon \Delta z$.

We performed a series of experiments under different sample concentrations in order to validate the applicability of the Beer-Lambert law. Rhodamine 6G (Sigma Aldrich 83697) was dissolved in deionized water and the concentrations used were 10, 50, 100, 250, 500, 1000, and 2500 μM . The power of the excitation light was measured by the power meter adjusted for the peak wavelength of the LED source, $\lambda = 469 \text{ nm}$. The power of the excitation light, which exited from the objective onto the sample, was 0.19, 0.36 mW, 0.53, 0.70 and 0.87 mW, respectively. This is shown in Fig. 5.3(a). The power of the excitation light measured adjacent to the light source was 0.45, 0.85, 1.23, 1.62 and 2.00 mW, respectively. The ratio between the light coming out from the objective and that coming out from the light source is around 43%. The positions of the solution slides were maintained the same throughout the experiments so that the absorption path lengths would be the same. As only Rhodamine 6G solutions were used in the experiments, the molar extinction coefficient was not changed. In another words, D was held constant.

5.3.5.3 Poisson distribution of the detected fluorescence emission light

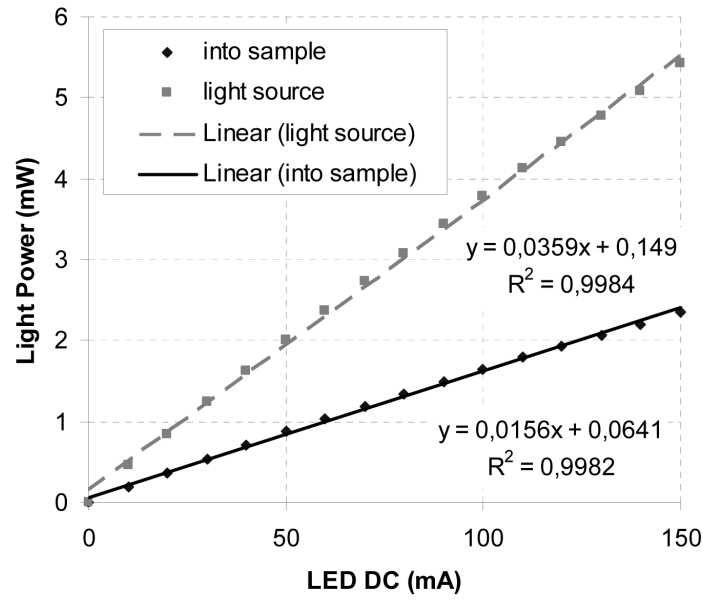
As a discrete probability distribution, the Poisson distribution describes the probability of a number of independent events (e.g. photon emissions) occurring in a fixed period of time on the condition that these events occur with a known average rate and independently of the time since the last event. The Poisson distribution is given in Eq. (5.22):

$$p(n|\mu) = \frac{\mu^n e^{-\mu}}{n!} \quad n = 0, 1, 2, 3, \dots \quad (5.22)$$

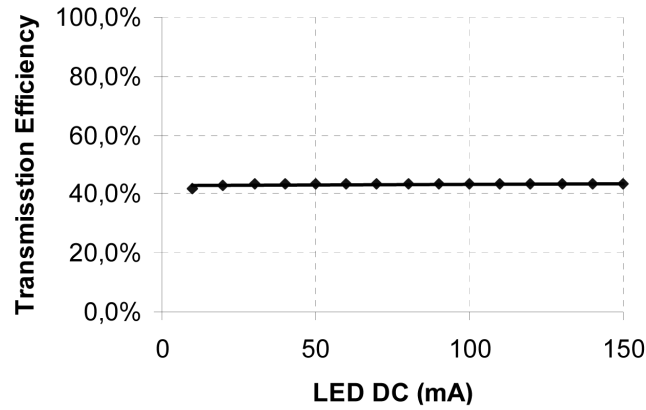
The expected number of photons that occur during the given interval is μ and the number of random occurrences of an event is n . Two important properties of the Poisson distribution (as used in Eq. (5.20)) are: (1) the average number of occurrences equals μ , i.e. $\langle n \rangle = \mu$, and (2) the variance is also equal to μ , that is, $\sigma_n^2 = \langle (n - \mu)^2 \rangle = \mu$.

In order to avoid photobleaching in a biology sample or a fluorophore solution, a green fluorescent plastic test slide (Lambert Instruments) was used in this measurement. Two images (i_1 and i_2) were taken consecutively with the microscope focused on the same place on the green fluorescent plastic slide under controlled LED DC current settings. The signal levels (per pixel) in these two images are denoted n_1 and n_2 . We now look at the difference between these two images, which represents the difference of two independent samples of one random process. This gives:

$$\langle n_1 - n_2 \rangle = \langle n_1 \rangle - \langle n_2 \rangle = 0 \quad (5.23)$$



(a)



(b)

Figure 5.3: Validation of the linearity of the entire measurement system and the constancy of the transmission efficiency of the optical components. (a) Light at the light source and the light exiting from the objective lens as the LED DC current is varied from 10 to 150 mA. Note that measured power is linear with the LED current. (b) Transmission efficiency of the optical component. Note that the efficiency is constant as a function of LED current.

In words, the mean value of the difference should equal the difference of the mean values per pixel in the two images. This, in turn, is zero as the two images were taken under the same LED DC current setting (Eq. (5.23)) and thus represent independent samples of the same random process.

The variance, however, equals the sum of the two noise variances per pixel in the two independent images (Eq. (5.24)). Until now we have made no use of an explicit distribution for the light intensities other than that they have a mean and variance. If we now assume that the distribution of the number of emitted photons is Poisson then we can make use of the explicit values for the mean and variance of such a process. Repeating the acquisition of pairs of images under differing intensities by varying the LED DC current settings (10 mA to 50 mA), this variance should be twice the average intensity.

$$\sigma_{n_1-n_2}^2 = \sigma_{n_1}^2 + \sigma_{n_2}^2 = 2\sigma_n^2 = 2\mu \quad (5.24)$$

5.4 Results and discussion

5.4.1 The power of the light source

If we assume a fluorescein molecule concentration of approximately $c = 2\mu\text{M}$, then there are $m \approx 11$ molecules per voxel; see Eq. (5.5). Allowing $r = 12$ phase recordings, each of which takes $T = 200$ milliseconds, and the time interval between two recordings is $T_0 = 0$ seconds, $n_{rec} = (30000 \times 11)/12 = 27500$ photons per recording can be used as a maximum value per voxel, Eq. (5.6). Absorbance $A = 1.74 \times 10^{-6}$ over one voxel path length, Eq. (5.9). We find that $n_0 = 7.61 \times 10^9$ excitation photons per voxel per recording are needed to obtain 3.06×10^4 absorbed excitation photons, Eq. (5.10). The number of photons $n_{0source}$ we need from the light source will then be 9.15×10^9 , Eq. (5.11).

If one recording takes 200 milliseconds, we have a maximum of $n_i = 512 \times 512 \times 9.15 \times 10^9 / 0.2 = 1.2 \times 10^{16}$ photons per second for illumination of the entire field of view, Eq. (5.12). This means a monochromatic 488 nm laser source ($= 4.07 \times 10^{-19}$ J/photon) with an optical power of about 5 mW is required for excitation of the entire sample, Eq. (5.13). At the sample plane the optical power that will be delivered at $\lambda = 488$ nm is given by $W_{sp} = (R_D \cdot \tau_{EF} \cdot \tau_{lens01}) \cdot W = (0.87) \cdot 5 \text{ mW} = 4.3 \text{ mW}$, from Eq. (5.13). The validity of the assumptions used in the model will be discussed later in this paper.

Using the same method, the excitation powers needed are given in Table 5.1 for other molecules, assuming that the parameters found in the literature are correct. If we require a certain SNR to achieve a required measurement precision for a parameter such as fluorescence lifetime, it might not be necessary to use the maximum number of photons. If, for example, a measurement precision of 1% is reached with half the number of photons that a molecule is capable of producing, then there is no need to use further illumination.

5.4.2 The SNR at the detector

Using Eq. (5.18) for one molecule, approximately 27 photoelectrons can be collected per image by the camera per phase recording when the total phase recording number $r = 12$. For every 100 emission photons, $n_e/n_{epr} \approx 1\%$, Eq. (5.18), which means that approximately one photon will be converted into a photoelectron. The SNR before ADC conversion will be $SNR \approx 27/(27)^{1/2} \approx 5 : 1 \approx 14$ dB, Eq. (5.20). With an electronic gain for the camera of $g = 0.126$ [ADU/e-] [99], an ideal estimation of the SNR for one molecule in an image is 5 (14 dB), which is good enough to eliminate the need for an electron multiplication (EM) readout for a charge-coupled device (CCD) camera system. By ideal we mean that, assuming all other noise sources are negligible, the SNR will only be limited by the Poisson-distributed, quantum photon noise. In the case of 15 dB or better, a typical high-end CCD without EM register performs better than a typical high-end EM-CCD, which adds multiplication noise [121]. But, should the excitation source be weaker, the quantum yield or the molar extinction coefficient be significantly lower, or the CCD be less sensitive to the emission wavelength, then EM could be required.

The SNR above is for one molecule in an image. Using a realistic estimate for a typical number of molecules (11 molecules per voxel), the power of a light source needed for FLIM is about 5 mW, and the expected SNR for a single camera pixel and for an entire image are 18 : 1 (25 dB) and 9000 : 1 (79 dB), respectively.

Using the parameter values found in the literature, the SNR for other fluorophores can be calculated, leading to the results shown in Table 5.2. In this table we present the SNR for 1) a single molecule, 2) a single pixel at a fluorophores concentration of $c = 2\mu\text{M}$, and 3) for an entire image at a concentration of $c = 2\mu\text{M}$.

5.4.3 Assumption and parameter validation

5.4.3.1 Transmission efficiency of the optical components

The results in Fig. 5.3(a) show that the system is linear, the light power both at the source and at the exit pupil of the objective lens increase linearly with an increased DC current to the LED. By dividing the light power at the exit pupil of the objective lens with the light power at the light source, the transmission efficiency of the optical component chains remains a constant, as shown in Fig. 5.3(b). The transmission efficiency (43%) is not high in this case due to the measurement configuration required for the laser power meter. A constant fraction of the photons was blocked before they could reach the exit pupil of the objective lens. But as the results show, we can treat the transmission efficiency of the optical components as a constant parameter in the mathematical model. Further, Fig. 5.3(a) tells us what current levels are required to achieve a given power level, W_{sp} , at the sample plane.

5.4.3.2 Influence of concentration on the fluorescence emission intensity

Fig. 5.4(a) shows the fluorescence emission light power as a function of solution concentration. Each data point is the average of three measurements for a given Rhodamine 6G concentration [μM] and LED current [mA]. The experimental data under differing LED DC current settings and differing concentrations fit well with the model in Eq. (5.21), the R-squared values are 0.9930, 0.9909, 0.9926, 0.9916, and 0.9916 under 10, 20, 30, 40, and 50 mA LED DC current, respectively. Fig. 5.4(b) is a plot of the value of B found by fitting Eq. (21) to the data averaged over all seven concentrations (10, 50, 100, 250, 500, 1000, and 2500 μM) under different LED DC current settings. This shows that the measured intensity parameter B is linearly related to the LED DC current. Fig. 5.4(c) is a plot of the value of D found by fitting Eq. (5.21) again averaged over the seven concentrations at each of the LED DC current settings. Fig 5.4(c) shows that D remains the same and is independent of the emission intensity as we expect. We conclude that the Beer-Lambert law is appropriate for obtaining the absorption factor over the range of Rhodamine 6G concentrations used here.

5.4.3.3 Poisson distribution of the detected fluorescence emission signal

The experimental results are shown in Fig. 5.5. Using the LED DC current setting at 10 mA as an example, Fig. 5.5(a) shows one of the two images acquired from the green fluorescent plastic sample. The difference between the two images, caused by the random noise, is shown in Fig. 5.5(b). By varying the LED DC current setting from 10 mA to 50 mA, the mean and the variance of the difference image for a given current setting can be plotted as a function of the LED DC current value. Fig. 5.5(c) shows that the mean value of the difference image under different current settings is close to zero as predicted in Eq. (5.23). Fig. 5.5(d) shows that the variance of the difference images increases linearly with the LED DC current value, as expected. Together, they validate the Poisson distribution assumption used in the mathematical model.

5.4.3.4 Final validation

The models and their associated equations given above have produced a variety of predictions for light source strength and SNR for varying fluorophores. The experiments presented above are intended to validate these models by testing measured values against predictions.

We have performed additional experiments to test the entire scheme using U2OS (osteosarcoma) cells that expressed GFP. The laser power meter was used to measure the excitation light intensity at the sample plane and using the result shown in Figure 5.3a we adjusted the LED DC current to produce $W_{sp} = 1.5$ mW of excitation light into each sample. This excitation power level was sufficient to produce high-quality images suitable for lifetime measurements. This value is significantly below the value of 94 mW in Table 5.1 because we did not try to extract the maximum number of photons from the GFP molecules. We also used an exposure time of 20 ms instead of the 200 ms in Table

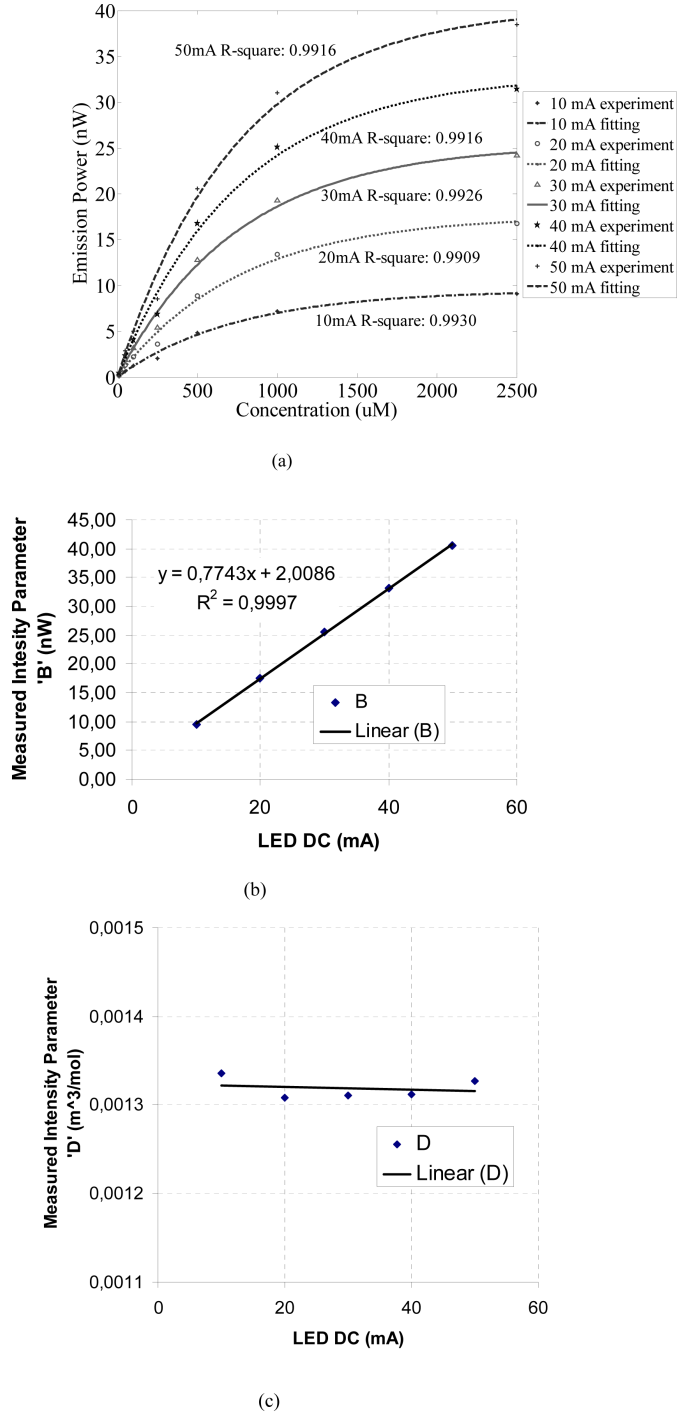


Figure 5.4: Influence of sample concentration, c [μM], on the fluorescence emission intensity. (a) the fluorescence emission light power as a function of solution concentration for different LED current settings; (b) the measured intensity parameter B from Eq. (5.21) as a function of LED DC current averaged over the seven different concentrations; and (c) the product of molar extinction coefficient and the absorption path length D from Eq. (5.21) averaged over the seven different concentrations.

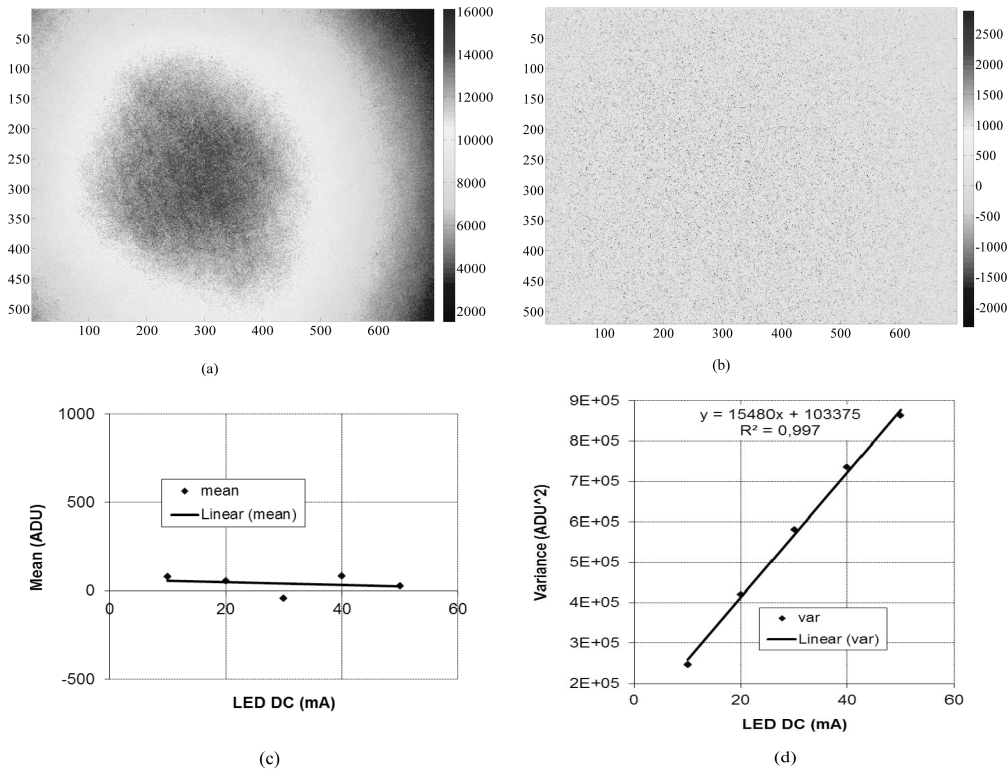


Figure 5.5: Poisson noise validation for the detected fluorescence emission light. (a) A single image taken from the green fluorescent plastic test slide at 10 mA; (b) The difference of the two “noise” images each acquired at 10 mA; (c) The mean value of the difference images as a function of LED DC current varying from 10 mA to 50 mA; and (d) The variance of the difference images as a function of LED DC current varying from 10 mA to 50 mA. It is this linearity that is indicative of the photon limited (Poisson) characteristic of the noise.

Table 5.3: Measurement results for U2OS cells expressing GFP. Experimental parameters were $\lambda_{ex} = 469$ nm, $NA = 0.5$, $n = 1.0$, $T = 20$ ms, and optical excitation power at sample $W_{sp} = 1.5$ mW. The predicted SNR is based upon Eq. 5.20.

Sample	Number of pixels	Average / pixel	Measured SNR / pixel	Predicted SNR / pixel
GFP slide - background	10×10	100.2	10.01 : 1 (20.0 dB)	
GFP slide - low intensity cell	10×10	167.3	12.93 : 1 (22.2 dB)	
GFP slide - middle intensity cell	10×10	759.7	27.56 : 1 (28.8 dB)	
GFP slide - high intensity cell	10×10	3746.9	61.21 : 1 (35.7 dB)	423 : 1 (52.5 dB)

5.1. We used the Olympus/LIFA system described in section 5.3.5.3 with the $20\times$ Zeiss objective lens with an $NA = 0.5$. For each cell, two images were acquired for the reasons described in section 5.3.5.3. In each pair of cell images a sample region was chosen. We then measured the SNR in that region. For each cell, we subtracted the contribution of the background variance from the total variance before we calculated the SNR per cell region. Our results are shown in Table 5.3.

The predicted SNR value is higher than the highest measured value by a factor of seven. The predicted value, however, was based upon the SNR that could be achieved if every single molecule in a pixel were illuminated until it had produced the *maximum* number of emission photons. This was not the case in our experiment. The samples we used were still very much “alive” after the images were recorded, that is, they were capable of producing more GFP emission photons.

Further, the wavelength dependence of the emitted photons and the assumption of wavelength constancy for various components as described in Eq. 5.19 can lead to an overestimate for the predicted SNR. Approximately 39% of the GFP photons, for example, have a wavelength outside the previously indicated (λ_1, λ_2) interval. Together, these two effects - less-than-maximum photon production and wavelength dependency - can explain the lower-than-predicted, measured SNR.

More importantly, with this amount of illumination delivered to the sample, the intensity values we measured were compatible not only with ordinary widefield fluorescence digital imaging but also with the requirements for lifetime imaging. Using the LIFA system and $W_{sp} = 1.6$ mW of optical excitation power, we measured a fluorescence lifetime for the GFP in the U2OS cells of $\tau_{\vartheta} = 2.17 \pm 0.14$ ns. This compares favorably with lifetime values around 2.1 ns reported in the literature [122] and shows that at this excitation power level a precision (CV) of 6.5% can be achieved in the measurement of the lifetime. These results demonstrate that our predictions over the entire system-from light source to digital image-are supported by these data.

5.5 Conclusions

A quantitative analysis has been made of the photon “budget” in a FLIM system. This concept is relevant to many fluorescence microscope users and the formulas are not restricted to FLIM but applicable to widefield fluorescence microscopy in general. For widefield fluorescence microscopy values to be determined, we need only set $r = 1$ in the various equations to determine the required excitation source power and the resulting SNR in the image. A light source of only a few milliWatts is sufficient for a FLIM system using fluorescein as an example. For every 100 photons emitted, around one photon will be converted to a photoelectron, leading to an estimate for the ideal SNR for one fluorescein molecule in an image as 5 (14 dB). The SNR for a single pixel and for the whole image with the molecule concentration of $2 \mu\text{M}$ are 18 (25 dB) and 9000 (79 dB), respectively. At this SNR the need for electron multiplication (EM) readout for a CCD camera system is dubious. But, as pointed out earlier, for any of a number of reasons—a weaker excitation source, a lower quantum yield or molar extinction coefficient, or a reduction in CCD sensitivity—the SNR could decrease which would mean that EM readout would be beneficial. Calculations of other fluorophores are also given as examples, such as Fura-2, green fluorescent protein (GFP), yellow fluorescent protein (EYFP), Rhodamine-6G, Alexa-546, Cy3, tetramethylrhodamine (TMR), and Cy5.

We have performed experiments to validate the parameters and assumptions used in the mathematical model. The transmission efficiency of the lenses, filters, and mirrors in the optical chain can be treated as a single constant parameter. The Beer-Lambert law is applicable to obtain the absorption factor in the mathematical model. The Poisson distribution assumption used in deducing the SNR is also valid. This quantitative analysis provides a framework for the design and fabrication of current and future Fluorescence (Lifetime Imaging) Microscope systems.

In this paper we have defined and used a large number of parameters, which are summarized in Table 5.4 together with their units, typical values (as used in this manuscript) and definitions.

Table 5.4: The names, units, values, and definitions of 41 parameters that are used in this chapter. The values are taken from the fluorescein example developed in this chapter.

Parameter	Units	Manuscript value	Meaning
λ_{ex}	[nm]	494	peak excitation wavelength
λ_{em}	[nm]	525	peak emission wavelength
τ_{ϑ}	[ns]	4.1	fluorescence lifetime measured from phase shift
τ_m	[ns]	4.1	fluorescence lifetime measured from modulation depth
V	$[\mu\text{m}^3]$	0.01	volume of one voxel
b	$[\mu\text{m}]$	25	linear size of one square pixel
a	-	512	number of pixels in row of square CCD image

Continued on next page

Table 5.4 – continued from previous page

Parameter	Units	Manuscript value	Meaning
M	-	$100\times$	magnification of objective lens
n	-	1.51	refractive index of immersion medium
NA	-	1.3	numerical aperture of objective lens
Δz	[nm]	147	depth-of-field
c	[mol/m ³]	0.2×10^3	molecule concentration (in moles)
m	[molecules/voxel]	11	molecules per voxel
T	[s]	0.2	exposure time of one image
T_0	[s]	0	time interval between two exposures with excitation illumination left on
r	-	12	number of (FLIM phase) images to be recorded
n_{emit}	-	30000	maximum number of photons / molecule emitted before photobleaching
n_{rec}	-	27500	number of photons / recording / voxel before photobleaching
Φ	-	90%	(emitted photos) / (absorbed photons)
n_{absorb}	-	30556	number of absorbed photons / recording / voxel before photobleaching
$\epsilon(\lambda_{ex})$	[m ² /mol] or [M ⁻¹ cm ⁻¹]	59668	molar extinction coefficient
n_0	-	7.6×10^9	number of excitation photons required to produce a given number of absorbed photons
$R_D(\lambda)$	-	95%	reflection coefficient of the dichroic mirror
$\tau_{EF}(\lambda)$	-	95%	transmission coefficient of the excitation filter
$\tau_{lens}(\lambda)$	-	96%	transmission coefficient of a lens in the excitation path
E_{ex}	[J/photon] or [eV/photon]	4.1×10^{-19} or 2.54	energy per photon from excitation source
W	[milliWatts]	5	optical power of excitation light source
W_{sp}	[milliWatts]	4.3	optical power of excitation light source at sample plane
SNR	ratio or [dB]	5:1 or (14)	signal-to-noise ratio after digitization
ϑ	[radians] or [°]	1.03 or 59°	half of the acceptance angle of objective lens
n_{epr}	-	2500	usable photons / recording / molecule
n_{lens}	-	625	number of photons that are collected by the objective lens / recording / molecule
γ	-	25%	% of emitted photons captured by objective lens
$\tau_D(\lambda)$	-	90%	transmission coefficient of the dichroic mirror
$\tau_B(\lambda)$	-	95%	transmission coefficient of the barrier filter
$\tau_W(\lambda)$	-	96%	transmission coefficient of the camera window

Continued on next page

Table 5.4 – continued from previous page

Parameter	Units	Manuscript value	Meaning
F	-	40%	camera fill factor
$\eta(\lambda)$	-	30%	quantum efficiency of the camera
κ	-	50%	area of CCD / area of illumination field
n_e	-	27	number of photoelectrons / molecule / recording
g	[ADU/e ⁻]	0.126	digital gray levels / photoelectron

5.6 Future works

In a future paper we will examine the estimation of fluorescence parameters, such as lifetime as a function of SNR and sample heterogeneity. As in this paper, results will be based upon a mathematical model and experimental results, but with the addition of simulations.

Considering the results obtained from the mathematical model and with the help of the simulation package, we are working on building a new type of FLIM system. The current implementation of frequency-domain FLIM requires an image intensifier based on a micro-channel plate (MCP) [6]. This conventional system has room for improvement and a robust solid-state camera would present a desirable alternative to MCPs [123, 124]. We are, therefore, designing and building a CCD image sensor that can be modulated at the pixel level.

The proposed FLIM system should have the following advantages: (1) there will be no need for a high voltage source, (2) the entire signal will be used during demodulation, (3) spatial resolution will be limited only by optics and pixel dimensions, (4) there will be no geometric distortion, and (5) as we have become accustomed with solid-state devices, it will be compact and relatively low cost.

5.7 Acknowledgement

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CHAPTER 6

MEM-FLIM architecture

Abstract

Our noncooled MEM-FLIM sensor has been designed for pixel-level modulation, which means that the demodulation is done on the camera pixel itself, instead of on an image intensifier, which sits in front of the CCD camera in the conventional method. In this chapter we present two architectures for MEM-FLIM cameras: one is a horizontal toggling MEM-FLIM camera (for simplicity, the “MEM-FLIM1” camera), the other is a vertical toggling MEM-FLIM (“MEM-FLIM2”) camera. The system schematic and experimental setup for both MEM-FLIM systems and a reference image intensifier based FD-FLIM system are presented, together with the lifetime procedure in the MEM-FLIM system. Finally we compare the hardware parameters of the MEM-FLIM cameras together with the intensifier based reference camera.

Part of chapter is based on publication on Journal of Biomedical Optics 17(12), 126020 (2012).

Keywords: CCD, pixel-level modulation, MEM-FLIM

6.1 Introduction

Given the disadvantages associated with the use of image intensifiers in conventional FD-FLIM, researchers start to look for alternative method for FD-FLIM. We are not the first group to use the approach of demodulation at the pixel level. In 2002, Mitchell et al [125, 126] demonstrated the feasibility of measuring fluorescence lifetime with a modified CCD camera. By modulating the gain of a CCD at a frequency of 100-500 KHz, images were recorded with an increasing delay. This camera, however, was not really suitable for FLIM since the maximum modulation frequency could only be 500 kHz. The “sweet spot” for frequency in an FD-FLIM system is approximately $f_o = 1/(2\pi\tau)$ which for $\tau = 5$ ns translates to about 30 MHz [2]. The value of 500 kHz is clearly too low.

In 2003, Nishikata et al. [127] succeeded in taking two phase images simultaneously at a modulation frequency of 16KHz. Again the modulation frequency is much too low but the two-phase approach can be found in our work as well.

Later Esposito et al [123, 128] developed this technique further and performed FLIM measurements at 20 MHz using a CCD/CMOS hybrid sensor (SwissRanger SR-2). The SR-2 was originally developed for full-field 3D vision in real time [129]. Later in this thesis, we will compare the performance of this camera to our implementation for frequency-domain FLIM.

Solid-state camera can also be used in TD-FLIM. The MEGA frame project, started in 2006, and is time-domain based. A complementary metal oxide semiconductor (CMOS) single-photon avalanche diode (SPAD) based camera has been developed for TD-FLIM [130, 131]. The prototype camera has 128×128 pixels.

6.2 Sensor architecture for MEM-FLIM cameras

We have designed and fabricated two types of MEM-FLIM cameras. Both MEM-FLIM cameras are front illuminated CCDs. The main principle of our designs is that the demodulation is done on the pixel level instead of on an image intensifier, which sits in front of the CCD camera in the conventional method. Demodulation signals, which have a 180-deg phase difference, are applied on two adjacent toggling gates of one pixel. In the first half of the demodulation cycle, the photo-generated charge will be transferred to one CCD vertical register (VR) adjacent to photo gate (PG) in horizontal toggling MEM-FLIM (MEM-FLIM1) or one storage gate (STG) in vertical toggling MEM-FLIM (MEM-FLIM2), and in the second half of the cycle to the other VR or STG, as shown in Fig. 6.1 and Fig. 6.2. In this way, two phase images are obtained in one integration and read-out cycle. So the readout image contains these two phase images interleaved with each other, the “phase one” image and the “phase two” image. The incoming light is thereby captured by modulated pixels, recording two phase images at once. This is in contrast to an image intensifier with a duty cycle of about 50% when recording a single phase image. By removing the intensifier and fiber/lens coupling from the camera, a noise source is eliminated as well as a source of image distortion. These two types of cameras

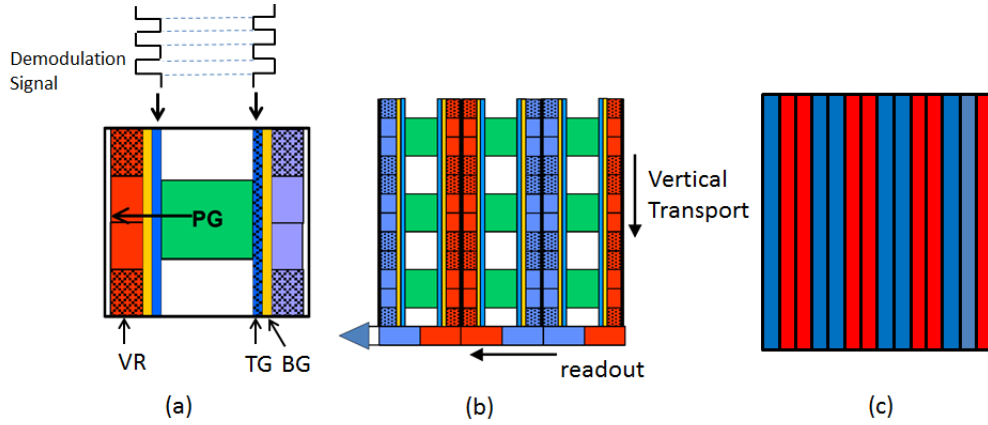


Figure 6.1: The principle of MEM-FLIM1 camera: (a) toggling principle at pixel level, (b) architecture of the chip level. BG: blocking gate; VR: vertical register; TG: toggling gate; and PG: photo gate, and (c) the illustration of two phase images interleaved with each other.

are based on the same principle and the difference lies in the technical implement. The detailed descriptions of these two architectures are as follows.

6.2.1 Horizontal toggled MEM-FLIM

The architecture of MEM-FLIM1 is similar to an interline CCD sensor, as shown in Fig. 6.1. The charge is collected and then transferred in the horizontal direction to either the left or the right VR adjacent to the PG on different phases of the modulation signal on the toggle gates (TG). The output pixel columns are in sets of two of the same phase image in order to minimize the capacitance and series resistance of the TG connection tracks, as shown in Fig. 6.1(c). The demodulation phase on the toggle gate is in the sequence of : $\dots 0^\circ - 0^\circ - 180^\circ - 180^\circ - 0^\circ - 0^\circ - 180^\circ - 180^\circ \dots$. Post processing is done in Matlab to recover the two phase images. Aluminum interconnects are used to shield the vertical registers from incoming illumination light. In the prototype we tested, however, the aluminum mask had a slight displacement (error) from its intended position. This meant that the photoelectrons that we measured were to a small extent caused by contributions from the wrong source. Between the register and the PG there was an extra blocking gate, the function of which is to prevent smear during reading out. The vertical registers shifted the phase images to the horizontal register after the exposure time during read-out. In MEM-FLIM1, dedicated registers were used to transfer the charge, which means there would be no smear effect if the light was left on during image transfer. The architecture complexity of MEM-FLIM1 limited its fill factor to 16%.

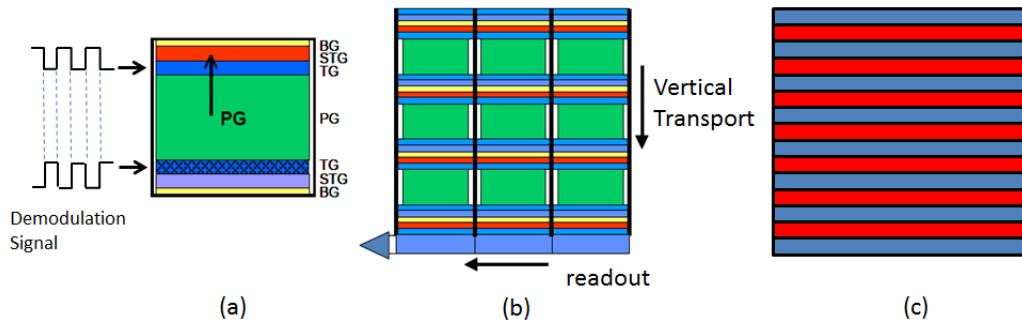


Figure 6.2: The principle of MEM-FLIM2 camera: (a) toggling principle at pixel level, (b) architecture of the chip level. BG: blocking gate; STG: storage gate; TG: toggling gate; and PG: photo gate, and (c) the illustration of two phase images interleaved with each other.

6.2.2 Vertical toggled MEM-FLIM

The architecture of MEM-FLIM2 is similar to a full frame CCD sensor, as shown in Fig. 6.2. The collected charge is transferred in the vertical direction to the STG either above or below the PG. The output pixel rows are interleaved with different phase, and the demodulation phase on the toggle gate is in the sequence of : $\dots 0^\circ - 180^\circ - 0^\circ - 180^\circ - 0^\circ \dots$. In the prototype we tested, the aluminum mask had a smaller area than intended. As a result, parts of the toggle gate were inappropriately exposed to the illumination light. This also means that photoelectrons that we measure are to a small extent caused by contributions from the wrong palce. Unlike the horizontal toggling design, there is no dedicated register for charge transfer during the readout in MEM-FLIM2. All the gates, including the photo gates, toggle gates, storage gates, and barrier gates are all used for vertical transport during read-out. This also requires the toggle gate clock to be off during vertical transport. The disadvantage of this design is that the light source needs to be switched off during the image transfer period, since the photo gate of the sensor is also used for charge transfer. This disadvantage of vertical design can be overcome by using a properly designed light source. MEM-FLIM2 has a higher fill factor (44%) compared to that of MEM-FLIM1(16%).

6.3 MEM-FLIM system

Both MEM-FLIM1 and MEM-FLIM2 have 212×212 active pixels, each of which is $(17\mu\text{m})^2$. The modulation frequency of MEM-FLIM1 was fixed at 20 MHz and that of MEM-FLIM2 at 25 MHz. The sensor sizes of both the MEM-FLIM1 and MEM-FLIM2 are the same ($4.9 \text{ (mm)} \times 5.3 \text{ (mm)}$). The wafer of the sensors is shown in Fig. 6.3(a) and the package of a single sensor is shown in Fig. 6.3(b). Figure 6.4 shows the camera board. The camera board is then put into an aluminum box and mounted on the right

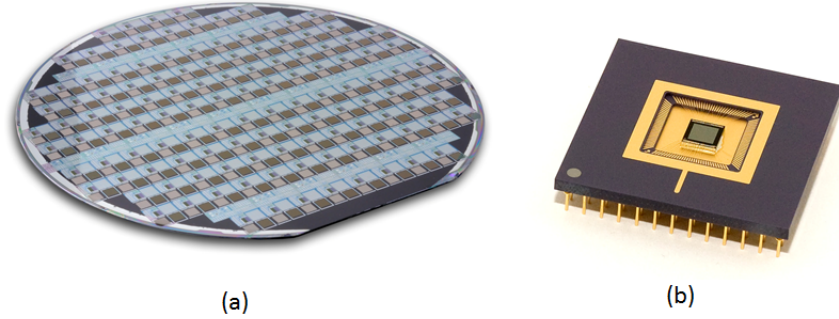


Figure 6.3: The image of MEM-FLIM sensor. (a) The wafer of sensor and (b) A single sensor after packaging.

side port of the microscope.

The schematic overview of the MEM-FLIM system setup with a wide-field microscope is shown in Fig. 6.5. The system is quite compact. There is no extra unit to generate a modulation signal for the LED; it comes from the MEM-FLIM camera itself. The experimental setup of the MEM-FLIM system is shown in Fig. 6.6. Our MEM-FLIM system includes an Olympus inverted microscope system IX-71 (Olympus), a MEM-FLIM camera (which can mount different sensor architectures), a power supply (CPX200, AIM-TTI Instruments) for the camera which is able to offer +6 V and -5 V, and a Dell computer installed with the Windows XP operating system, Labview 8.5., Matlab 7.9.1 (R2009b) and LI-FLIM software version 1.2.6 developed by Lambert Instruments.

The interface for controlling the MEM-FLIM camera is shown in Fig. 6.7. Figure 6.7(a) shows the camera control panel, in which there are many subpanels. Our MEM-FLIM system has been designed with a variable integration time T_0 such that $1 \text{ ms} \leq T_0$. The choice of T_0 is related to the strength of the fluorescent image. The image is then read out before the next integration cycle begins. The time for integration plus read-out time T_R plus a user-chosen delay T_{DL} is referred to as the frame time T_1 , that is, $T_1 = T_0 + T_R + T_{DL}$. In the camera control panel, users are able to change the integration time T_0 and the frame time T_1 . Users can also adjust the analog gain and the phase delay between the LED and the camera in this panel. Figure 6.7(b) shows the frame grabber control panel which performs image visualization, capture, and save. Figure 6.7(c) and Fig. 6.7(d) are subpanels from Fig. 6.7(b). Figure 6.7(d) shows the real time image. One can choose to plot the intensity in one row or column from the real time image in Fig. 6.7(c), in this way, we can see whether the emission intensity is sufficient or whether the camera is saturated. Using the panels described above, one can take fluorescence images at different phases by changing the phase delay in the camera control panel and save the image in the frame grabber control panel. To change the phase delay like this, however, is too slow and inconvenient. The panel shown in Fig. 6.7(e) is designed to perform phase

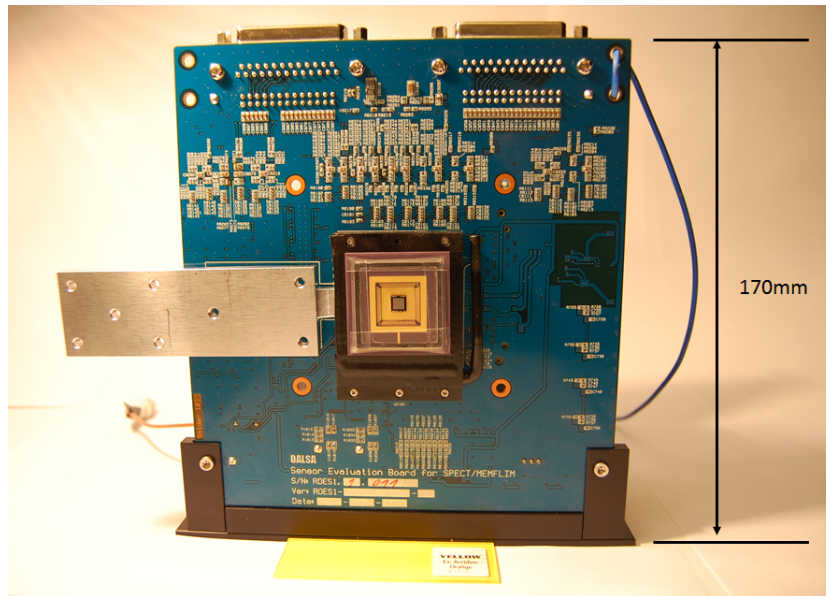


Figure 6.4: The image of the camera board of MEM-FLIM1 and MEM-FLIM2.

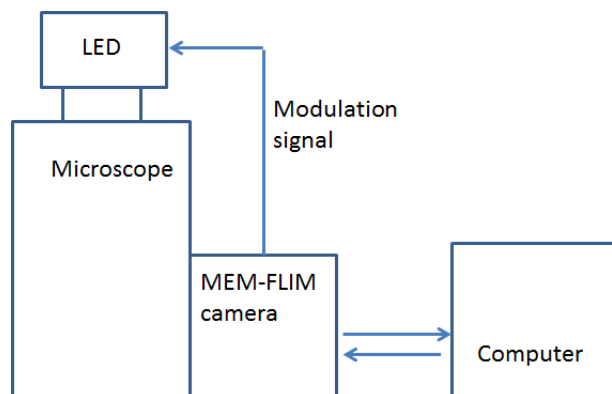


Figure 6.5: The schematic overview of the MEM-FLIM system setup with wide-field microscope.

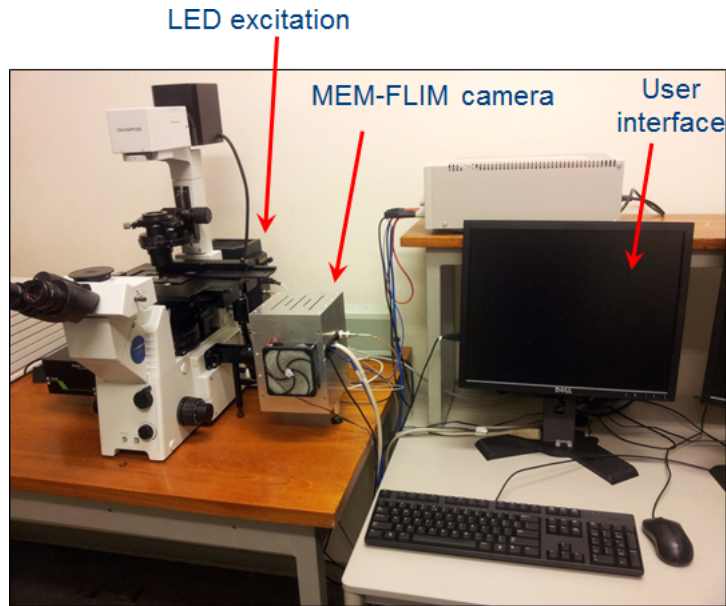


Figure 6.6: The image of experimental setup of the MEM-FLIM system.

changing and imaging saving automatically. In this panel, the user can set the number of phases in one lifetime image, the number of lifetime images, resting time between two lifetime images (in order to perform timelapse measurement) etc..

The work flow for doing FLIM measurement is shown in Fig. 6.8. A certain number of raw phase images together with a dark image are taken automatically via Labview interface. Each of these raw images contains two phase images, which are separated and arranged in sequence in Matlab. The dark image is used for background correction, which is also done in Matlab. Afterwards a “.fli” file is generated in Matlab from the sorted phase images and opened in LI-FLIM software. Finally, a region of interest can be chosen and data analyses can be done in LI-FLIM software.

6.4 Reference system

In order to evaluate the performance of a MEM-FLIM system, we need a standard conventional image intensifier based FD-FLIM system to serve as a baseline for comparison. The schematic overview of the reference system setup with wide-field microscope is shown in Fig. 6.9. Our reference FLIM system includes an Olympus inverted microscope system IX-71 (Olympus), a LIFA system (Lambert Instruments, Roden, The Netherlands) which includes a LI²CAM Intensified CCD camera (GenII with S25 photocathode) as the reference camera (Lambert Instruments, Roden, The Netherlands) and a Dell computer installed with the Windows XP operating system. The experimental setup is shown in Fig. 3.5. The reference system and the MEM-FLIM system share the light source and the microscope and have the same light path until the sample emission light is directed

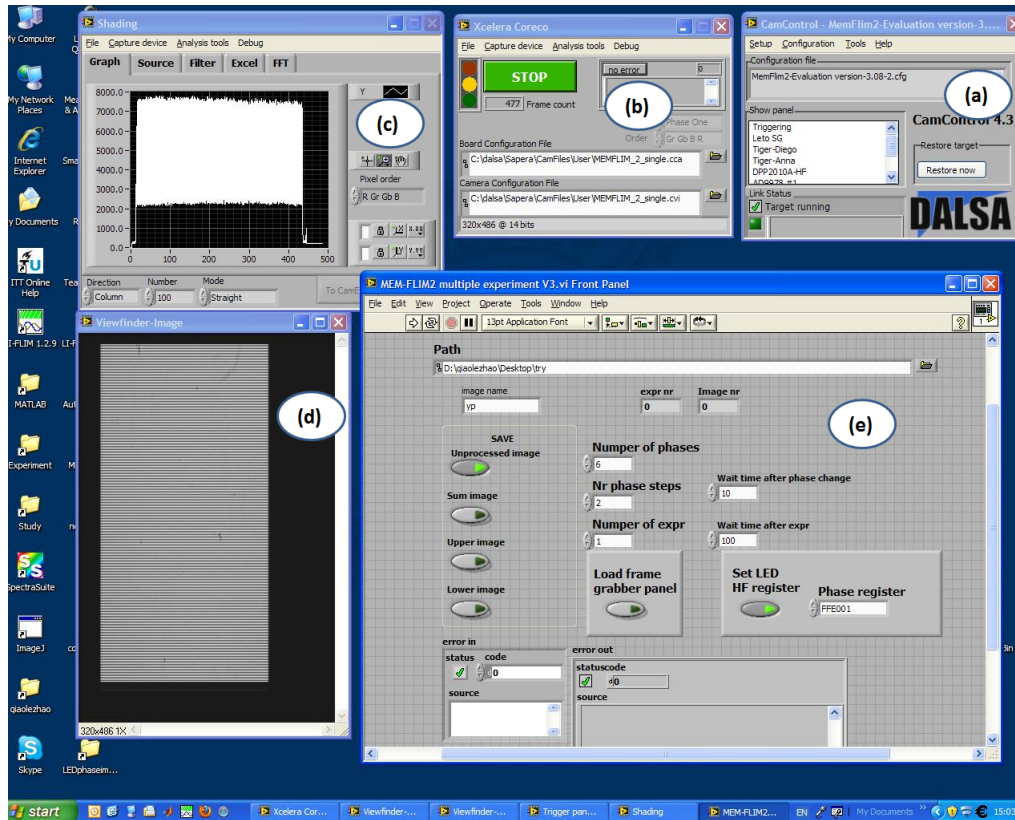


Figure 6.7: The interface for controlling the MEM-FLIM camera. (a) Camera control panel, (b) frame grabber control panel, (c) subpanel from (b) which can visualize the real time image, (c) subpanel from (b) which can plot intensity for a row/column pixel, and (e) automated panel for taking lifetime images.

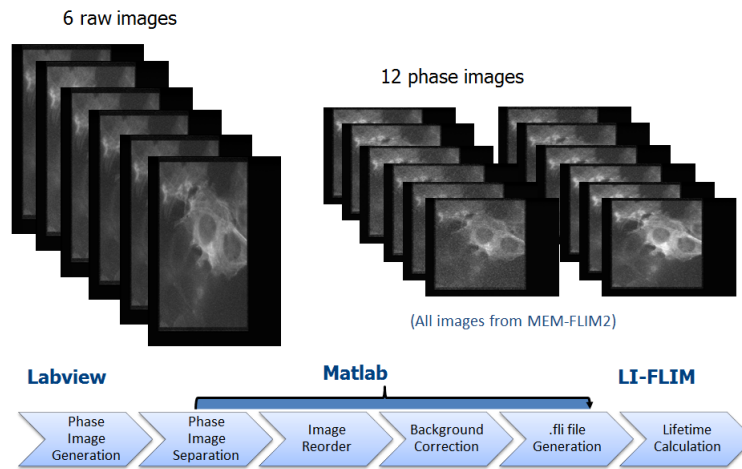


Figure 6.8: The schematic work flow for FLIM experiment using MEM-FLIM system. The images in this figure are taken from MEM-FLIM2.

into different cameras. When doing comparison experiment, the emission light from the sample is directed into either the MEM-FLIM camera or the reference camera, while the rest of the system remains the same. Comparing with the MEM-FLIM system, we can see the reference FLIM system has a bulky unit, which is used to control and supply high voltage to the image intensifier. The modulation signals for the camera and the LED are generated by this control unit, while in a MEM-FLIM system, the MEM-FLIM camera is controlled directly by the computer and the signal for the LED is supplied by the MEM-FLIM camera itself.

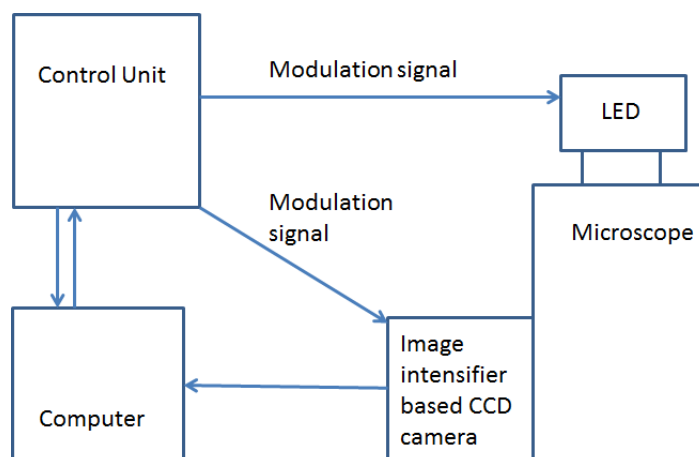


Figure 6.9: The schematic overview of the reference system setup with wide-field microscope.

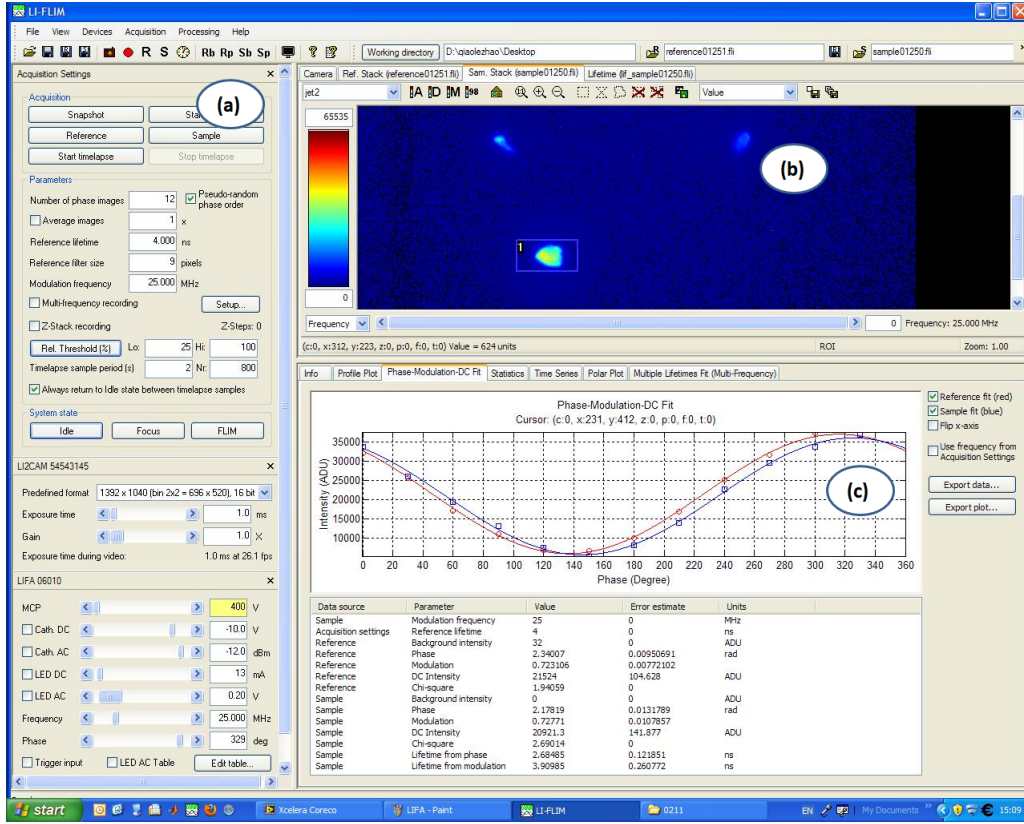


Figure 6.10: The interface for controlling the reference camera. (a) Hardware view, (b) data view and (c) information view.

The reference FLIM system is controlled via LI-FLIM software version 1.2.6 developed by Lambert Instruments. The interface of LI-FLIM software is shown in Fig. 6.10. The acquisition parameters such as modulation frequency, reference lifetime, and timelaps parameters can be set in Fig. 6.10(a). Hardware is also controlled here, such as voltages for the micro channel plate in the image intensifier, camera exposure time, LED modulation signal. Figure 6.10(b) is the visualization of the real time image, one can choose a region of interest and the analyzed data such as modulation depth, phase information, calculated lifetime etc. are shown in Fig. 6.10(c).

6.5 Conclusion

The comparison of MEM-FLIM1 and MEM-FLIM2 camera is shown in Table. 6.1. Both architectures do not have an EMCCD for signal amplification. Since in the future we will compare MEM-FLIM camera with a reference CCD camera which is used in conventional image intensifier based FD-FLIM system, we also list here the data from this reference camera. From the schematic and experimental setup, we can see that

MEM-FLIM system is a more compact and convenient system compared to the reference system.

Table 6.1: Design comparison of the MEM-FLIM cameras and the reference camera.

	MEM-FLIM1	MEM-FLIM2	Reference camera
Fill factor	16%	44%	>50%
CCD pixel size (μm)	17	17	20.6 ¹
Active pixel number	212×212	212×212	696×520
Modulation frequency (MHz)	20	25	0.001-120
ADC readout frequency (MHz)	20	25	11
Full well capacity (ke^-)	38	38	18
Bits	14	14	12

¹ The pixel size of the CCD sensor itself is $6.45 \mu\text{m}$, we are using 2×2 binned mode, which gives $12.9 \mu\text{m}$, and the pixels as “projected” onto the photocathode by the fiber optic taper are magnified $1.6 \times$, arriving at $20.6 \mu\text{m}$ of effective pixel size of the intensified camera system.

CHAPTER 7

MEM-FLIM evaluation technique

Abstract

In this chapter, parameters which describe the camera performance are introduced, such as linearity, sampling density, dark current, readout noise, sensitivity, etc. together with the methods of quantitatively measuring these values. MEM-FLIM cameras are evaluated using the evaluation methods described in this chapter. The results of the camera evaluations are presented in the next chapter. The parameters and methods described in this chapter are not only applied to our MEM-FLIM cameras and reference camera, but can also be used to evaluate other CCD cameras. FD-FLIM system calibrations before measuring fluorescence lifetime of samples are also presented here. The calibration allows one to quantify the phase change and the modulation change introduced by the system itself.

This chapter is based upon and extended from the publication in the Journal of Biomedical Optics 17(12), 126020 (2012).

Keywords: camera characteristics, evaluation technique, calibration

7.1 Camera characteristics - Background

7.1.1 Charge transfer efficiency

After a CCD pixel converts photons to electrons, the electrons are then transferred to the horizontal register and then pixel by pixel to the output unit. In the process of transferring, however, not every electron will be carried along. Due to the imperfect charge transfer efficiency (CTE), some of the electrons will be lost. CTE is the quantitative indicator of the device's ability to transfer charge from one potential well to the next. CTE is defined by R. Janesick as the ratio of the charge transferred from the one pixel to the initial charge stored in the that pixel [132], and expressed in Eq. (7.2). Equation (7.2) can be further simplified by using Poisson's approximation to the binomial distribution to Eq. (7.1). n is the trailing pixel number that follows the target pixel, N_p is the number of pixel transfers, S_i is the initial charge in the target pixel and S_{N_p+n} is the charge in the $N_p + n$ trailing pixel.

$$S_{N_p+n} = \frac{S_i N_p!}{(N_p - n)! n!} CTE^n (1 - CTE)^{N_p - n} \quad (7.1)$$

$$S_{N_p+n} = \frac{S_i (N_p (1 - CTE))^n}{n!} \exp(-N_p (1 - CTE)) \quad (7.2)$$

When measuring CTE we clock out empty lines after image region. An example is shown in Fig. 7.1. The camera is set to a long integration time without receiving light. Assuming the dark current charge of the first empty column has to travel through 227 register cells. The last image column has an intensity of $2600 - 200 = 2400$ (ADU), where 200 (ADU) is the average empty level calculated from the empty pixels, and 2600 (ADU) is the original intensity of the last image column. In the same way, the first empty column has an intensity of $600 - 200 = 400$ (ADU), where 600 (ADU) is the original intensity of the first empty column. With $n = 1$ and $N_p = 227$, Eq. (7.2) is then simplified to $400 = 2400 * (227 * (1 - CTE)) * \exp(-227 * (1 - CTE))$. The CTE can be calculated to be 0.9991.

7.1.2 Linearity of photometric response

It is extremely convenient for a scientific camera to have a linear response to the incident light, especially when applied for quantitative photometric analysis. As converting photons which carry image information to the electronic signal is the fundamental function of a CCD camera, the digitized output signal *Output* should be linearly proportional to the amount of photons that have reached the sensor N_{photon} , as shown in Eq. 7.3. G is the conversion factor from photons to ADUs, is the β readout noise in ADUs. Nonlinear performance of a CCD camera indicates that the gain of the camera is different at different signal intensities, which is not desired for quantitative image operations and algorithms that rely on absolute signal measurements such as linear transformations, shading correc-

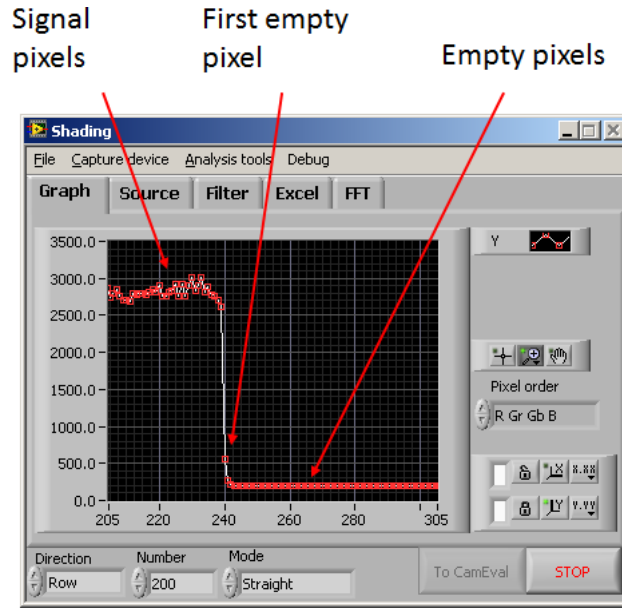


Figure 7.1: The illustration of calculating CTE.

tions etc. The CCD itself and with other electronic components in the signal processing chain determine the linearity of a camera system.

$$Output = \alpha N_{photon} + \beta \quad (7.3)$$

One needs to know whether and when the CCD is producing a linear photometric response. A commonly used technique for evaluating the CCD linearity is based on a graphical plot of measured signal intensity as a function of exposure time. The linearity of photometric response of a camera is gauged by the coefficient of regression, calculated from a straight-line fit of intensity readout data under various exposure times. The closer the coefficient of regression is to 1, the better the linearity of the camera. Below saturation, the CCD is usually photometrically linear. At high illumination intensity levels, a nonlinear response will be observed after the camera reaches the full well (saturation) condition.

7.1.3 Sampling density

Sampling in signal processing refers to the conversion of a continuous signal to a discrete-time signal: a sequence of samples. The Nyquist-Shannon sampling theorem shows that the criterion to reconstruct the original analog signal perfectly from the sampled version is that the sampling frequency should be greater than twice of bandlimit (highest frequency) of the original input signal. Under sampling may cause artifacts and image information will be lost.

Sampling density refers to the physical scale between pixels in the digitized microscope image, which establishes a direct connection between one pixel in the image and a real

size in the physical space. It describes the image acquisition condition and is determined by the configuration of the imaging system (magnification and quality of the objective and the detector pixel size).

An image with $a \times a$ pixels that covers a physical area at the specimen plane of $L \times L \mu\text{m}^2$ has a sampling density of a/L samples per micron in both directions. Equivalently, the sample distance along any of these directions is $L/a \mu\text{m}$. The sampling densities along both the horizontal and the vertical directions are preferably the same [34]. The sampling densities of the MEM-FLIM camera and the reference camera are measured by using a stage micrometer. A $20\times$, 0.5 NA objective lens is used in the experiment.

7.1.4 Resolution

Due to inevitable aberrations and diffraction phenomena, the image of an object observed with an optical system will be somewhat degraded. As a rule, the bright areas in the image will not be as bright as in the original pattern and dark area will be not as dark as in the original pattern. There will be a smooth transition along originally high contrast edges. The optical transfer function (OTF) is a commonly used quantity for describing the resolution and performance of an optical system [94].

One way to measure the OTF is to use a test pattern such as that shown in Fig. 7.2(a) and the OTF can be calculated from the edge response [34]. The procedures for obtaining OTF data used in this thesis are as follows: (1) choose a suitable region where the intensity goes from white to black. (2) Flat field correction is performed to get rid of possible shading due to non-uniform illumination, non-uniform camera sensitivity, and dark current etc.. The correction is done by using a “black” image which is taken when the camera shutter is closed and a “white” image with the camera focused on an empty field. The correction is done as shown in Eq. 7.4, and the resulting pixel values are between 0 and 1. (3) To prepare for the derivative operation in the following step, an interpolation is done in the horizontal direction to a sample spacing eight times finer using a spline interpolation routine on the corrected image. (4) A line response is generated from the edge response by using a 1-D derivative-of Gaussian kernel with coefficients ($\sigma = 1.5$) convolved with the interpolated image along each horizontal line. (5) The Fourier transform of each line response can now be computed to yield the estimate of the OTF in the horizontal direction. (6) Since the edge response is not perfectly aligned due to the manufacture of the test pattern, an averaged Fourier transform of certain number (N) of line responses is calculated by using the average of the sum of the absolute values of the Fourier coefficients of different lines at the corresponding frequencies (Ω), as shown in Eq. (7.5). The averaging over N lines improves the signal-to-noise ratio by a factor of $\sqrt{N}$. Eq. (7.5) works when the noise can be neglected, which in this case, indicated by the OTF value at high frequencies (>2000 cycles/mm) close to zero as shown in Fig. 7.2. When considering noise, Eq. (7.5) can be written as (7.6), which results the OTF tail values at high frequencies have an offset indicated by the noise. (7) Normalize OTF so that at zero frequency the OTF equals to 1. The fact that OTF is not equal to 1 at the zero frequency is due to the photon loss between the input illumination and measurement

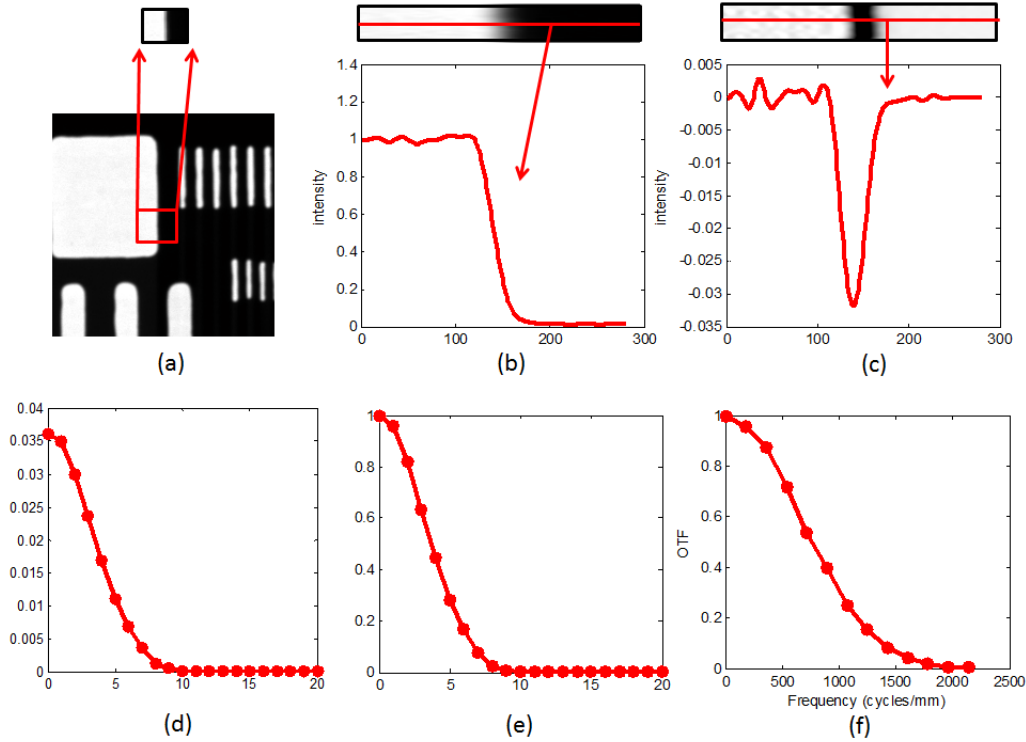


Figure 7.2: The procedure for measuring OTF from a edge response. (a) test pattern used in the experiment, (b) interpolated line profile of a step response, (c) line profile of a line response (differentiated edge response), (d) Fourier transform of the averaged line response, (e) Normalized OTF, (f) Mapping the x label to the unit of cycles/mm.

system, the amount of which is difficult to determine. (8) With the knowledge of pixel size of the CCD camera, the frequency unit is mapped into “cycles/mm”.

$$image_{corrected} = \frac{image_{original} - image_{black}}{image_{white} - image_{black}} \quad (7.4)$$

$$X_{average}(\Omega) = \frac{1}{N} \sum_{n=1}^N |X_n(\Omega)| \quad (7.5)$$

$$X_{average}(\Omega) = \left| \frac{1}{N} \sum_{n=1}^N X_n(\Omega) + \epsilon \right| \quad (7.6)$$

Our measurements are made in both the horizontal direction and the vertical direction. A higher OTF indicates a better performance of an optical system. The MEM-FLIM and reference FLIM systems share the same system settings (microscope, filter cube, illumination) except that the fluorescence emission can be switched and directed between

the two different camera ports. Thus the OTF directly reflects the performance of the camera. All OTF measurements have been made with a magnification of $100\times$, 0.6 NA oil objective lens and a 180 ms integration time. The test pattern was illuminated via transmitted white light.

The OTF can be influenced by effects such as the misdiffusion of the electrons generated outside the depletion layer, nonideal charge transfer effects, the photosensitivity of the device, and so on [133].

7.1.5 Noise

The main noise sources for digitized fluorescence images can be characterized as: photon noise due to the random arrival time of photons, dark current noise due to random generation of electrons by thermal vibrations, readout noise due to the on-chip amplifier which converts the electrons into a change in analogue voltage, and quantization noise due to quantizing the pixels of a sensed image into a number of discrete levels.

7.1.5.1 Photon noise

The fundamental quantum physics of photon production determines that the photon noise N_p is Poisson distributed [134], as shown in Eq. 7.7.

$$p(n|\mu_p) = \frac{\mu_p^n e^{-\mu_p}}{n!} \quad n = 0, 1, 2, 3, \dots \quad (7.7)$$

where μ_p is the expected number of photons during a given interval, and n is the number of random occurrences. To validate the Poisson distribution assumption, we make use of an important characteristic of the Poisson distribution: $\langle N_p \rangle = \mu_p = \sigma_p^2$.

The Poisson distribution assumption of the photon noise will be validated using the following method. Two (independent) images are taken under the same illumination condition. The photon noise level is determined by subtracting these two images so that deterministic pixel variability in the image (e.g. shading) can be eliminated. The total intensity variance of the difference image is the sum of the variances of the two independent images. As the two images have identical statistics, half of the variance in the difference image is variance of a single image. To confirm the assumption that the noise source of the camera is Poisson distributed, we take two images and obtain the difference image under different illumination intensities, that is, different average intensities. The variance for a Poisson distribution should be linear with the mean intensity [135].

7.1.5.2 Dark current noise

Dark current noise N_d refers to the creation of electron-hole pairs due to thermal vibrations [99]. It is intrinsic to semiconductors and is a stochastic process with a Poisson distribution and thus $\langle N_d \rangle = \mu_d = \sigma_d^2$. It reduces the dynamic range of the camera since it produces an offset to the readout value and it can be a substantial source of noise. Cooling the camera reduces the dark current significantly.

The dark current can be influenced by the previously defined integration time (T_0) and frame time (T_1) in the MEM-FLIM camera, and it is, therefore, necessary to evaluate their individual contributions. This can be accomplished by varying the aforementioned T_{DL} . The linearity of the dark current noise in the integration time is also validated using the same method as in Section 7.1.2. Since the name “dark current” refers to the electron-hole pairs that are created when the camera is not exposed to light, measuring dark current is relatively simple and requires no optical setup.

7.1.5.3 Readout noise

Readout noise N_r is a fundamental trait of CCD cameras caused by the CCD on-chip electronics in the process of reading the signal from the sensor before digitizing. It is independent of integration time but dependent on the readout bandwidth. By measuring the linearity of the dark current noise to the integration time, the readout noise with a mean of $\langle \mu_r \rangle = 0$ and a variance σ_r^2 can be deduced from the fitting by extrapolating the noise level in the limit as the integration time goes to zero. When the integration time is zero, photon noise and dark current noise are both zero, leaving only the readout noise.

7.1.5.4 Quantization noise

Quantization noise N_q is the round-off error when the analog-to-digital converter (ADU) converts a sensed image to a finite number of discrete levels, and thus $\langle N_q \rangle = 0$ and $\langle N_q^2 \rangle = \sigma_q^2$. Quantization noise is inherent in the quantization process. For a well-designed ADC with the number of bits b higher than 8 (the MEM-FLIM camera has 14 bits, and the reference camera has 12 bits), the quantization noise can be ignored as the signal-to-noise ratio (SNR) is given by $6b + 11$ dB [2, 99, 135].

7.1.6 Sensitivity

Sensitivity relates the A/D converter units (ADU) of a digital camera system to the number of photo-electrons produced by incident photons reaching the pixels.

7.1.6.1 Sensitivity

Sensitivity measures a camera’s ability to convert photo-electrons to ADUs. For a photon-limited signal, the conversion factor G from photo-electrons to ADUs can be determined by Eq. 7.8 [99]:

$$G = \left(\frac{\text{var}(I_1 - I_2)}{2} \right) / \bar{I} \quad (7.8)$$

I_1 and I_2 are two images taken under the same illumination condition. $\bar{I}$ is the mean intensity over a uniformly illuminated field. G , in the unit of ADU/e⁻, is indicated by the slope of the fitted linear curve to the photon noise measurements (section 7.1.5.1) for different intensities.

7.1.6.2 Detection limit

The “sensitivity” of a camera can also be described by the minimum light that can be detected. When the detected signal is smaller than the noise floor of the camera, the signal will be buried in the noise. Thus the noise floor, such as readout noise and dark current noise, determines the limits of the camera sensitivity. Assuming the photon noise is Poisson distributed, the mean of the minimum signal above the noise floor σ_n is μ_s and the standard deviation of the signal is $\sigma_s = \sqrt{\mu_s}$. We note that σ_n^2 is composed of several *independent* terms $\sigma_n^2 = \sigma_s^2 + \sigma_d^2 + \sigma_r^2 + \sigma_q^2$.

When the integration time T_0 is small, the noise floor σ_n is determined by the readout noise σ_r of the camera. We assume that the requirement for a signal not being buried in the noise floor is that the difference between the signal level and the noise level is at least k times bigger than the standard deviation of the signal, Eq. (7.9):

$$\begin{aligned} \mu_s - \sigma_n &\geq k\sigma_s \Rightarrow \\ \mu_s - k\sqrt{\mu_s} &\geq \sigma_n \Rightarrow \\ (\sqrt{\mu_s} - \frac{k}{2})^2 &\geq \sigma_n + \frac{k^2}{4} \Rightarrow \\ \mu_s &\geq \sigma_n + \frac{k^2}{2} + k\sqrt{\sigma_n + \frac{k^2}{4}} \end{aligned} \quad (7.9)$$

At a longer integration time, the influence of the dark current noise can not be ignored since the dark current noise σ_d increases with the integration time T_0 . Concurrently, the signal level is also increasing linearly with the integration time. If we note that given an integration time T_0 , the Poisson character of the photon signal and the dark current means that $\mu_s = v_s T_0$ and $\sigma_d = \sqrt{v_d T_0}$, respectively. We assume that the signal can be distinguished from the noise floor if the range of the signal does not overlap with the range of the noise, which gives us Eq. (7.10). Thus when the rate of electron generation (v_s and v_d) meets the condition in Eq. (7.10), the signal will be above the noise floor and can be detected by the camera.

$$\begin{aligned} \mu_s - k\sigma_s &\geq \mu_d + k\sigma_d + \sigma_r \Rightarrow \\ v_s T_0 - k\sqrt{v_s T_0} &\geq v_d T_0 + k\sqrt{v_d T_0} + \sigma_r \Rightarrow \\ v_s &\geq v_d + k\sqrt{\frac{v_d}{T_0}} + \frac{\sigma_r}{T_0} + \frac{k^2}{2T_0} + \frac{k}{\sqrt{T_0}} \sqrt{v_d + k\sqrt{\frac{v_d}{T_0}} + \frac{\sigma_r}{T_0} + \frac{k^2}{4T_0}} \end{aligned} \quad (7.10)$$

It is clear from this result that for long integration time ($T \rightarrow \infty$), the signal can be detected if:

$$v_s \geq v_d + 2k\sqrt{\frac{v_d}{T_0}} \quad (7.11)$$

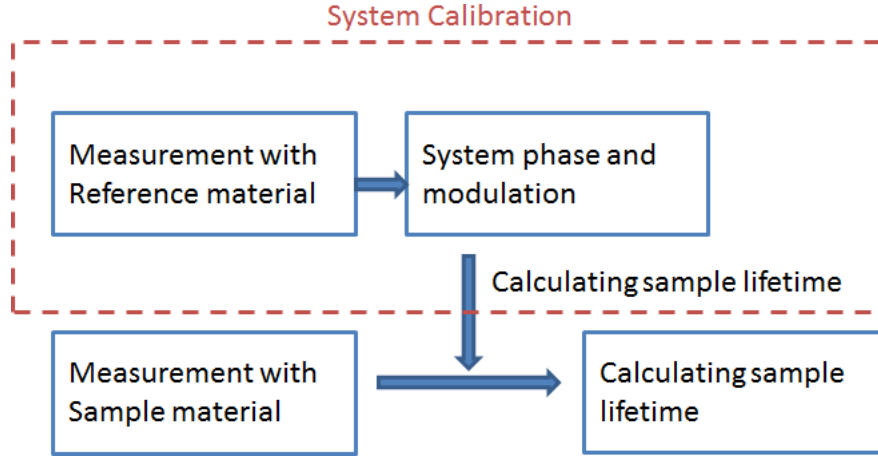


Figure 7.3: The workflow of measuring a sample with unknown lifetime.

7.2 System calibration of FD-FLIM

7.2.1 Method

For measuring a sample with unknown lifetimes, the FD-FLIM system has to be first calibrated in order to know the phase change and the modulation change introduced by the system itself. This has to be done for both the reference FLIM system and MEM-FLIM system. The workflow of measuring a sample with unknown lifetime is shown in Fig. 7.3. The way to calibrate the system is to calculate the phase change ($system_{phase}$) and the modulation depth change ($system_{modulation}$) of the system by using a reference fluorescent sample which has only one lifetime component with known lifetime as $refTau$, as shown in as shown in Eq. (7.12). $REFphase$, $REFmodulation$ are the measured phase and modulation of the known lifetime reference. Then the changes introduced by the system will be used when measuring the sample with unknown lifetimes, as shown in Eq. (7.13). $SAMphase$, $SAMmodulation$ are the measured phase and modulation of the unknown lifetime sample. ω equals to $2\pi f$ where f is the modulation frequency in Hz.

$$\begin{aligned} system_{phase} &= REFphase - atan(\omega * refTau) \\ system_{modulation} &= REFmodulation / (1 / sqrt((\omega * refTau)^2 + 1)); \end{aligned} \quad (7.12)$$

$$\begin{aligned} tau_{phase} &= 1/\omega * tan(SAMphase - system_{phase}) \\ tau_{mod} &= 1/\omega * sqrt(1 / (SAMmodulation / system_{modulation})^2 - 1); \end{aligned} \quad (7.13)$$

7.2.2 System stability

In order to know whether this calibration needs to be done before every lifetime measurement or can be done only once a day, the system stability was examined by measuring

a known lifetime (2.8 ns) fluorescent plastic slide[135]. The experiment was done on the MEM-FLIM2 camera with the modulation frequency of 25 MHz. The different intensities $I(\varphi)$ at 12 different phases are fitted with a sine signal to extract the parameters of the phase and the modulation, as shown in Eq. 7.14 and Fig. 3.4 in section 3.2.1. DC is the amplitude of the signal, φ is the controlled phase of the demodulation signal to the excitation signal, m and θ are the estimated modulation depth and phase.

$$I(\varphi) = DC(1 + m \cdot \cos(\varphi - \theta)) \quad (7.14)$$

The experiment was repeated for 36 times in 6 hours, and the extracted phase and modulation values were compared and analyzed. The whole system was turned on (including the LED power and the camera power) and was not switched off between experiments. Even though the bleaching for the plastic slide can be neglected, a mechanical shutter was used to prevent long time illumination between the experiments in order to prevent unnecessary heating of the slide during the 6 hours. This shutter was only open before each experiment. The results showed that the phase and the modulation parameters were quite stable with small changes of 0.3% and 0.9%, respectively. This means the phase and the modulation introduced by the system is quite stable, and the calibration can be done at the beginning of the experiment day.

This conclusion, however, is based on the system not being switched off between the experiments. If the system is switched off between experiments, even though the experiments are done after switching on the system and allowing a certain time for the system to stabilize, the changes introduced by the system can be quite different for each experiment. Experiments were done using the same setup and material as above (the only difference is whether to switch off the system), and the phase change can result in a 16.2% difference while the modulation change is 2.3%. The bigger change in the phase than in the modulation is due to the instability of the LED after being switched on. The heating up of the LED influences the phase change quite a lot until the LED reaches a stable state.

CHAPTER 8

MEM-FLIM evaluation results

Abstract

The MEM-FLIM1 and MEM-FLIM2 cameras are evaluated using the method described in the last chapter. The results of the evaluation are presented and discussed in this chapter. The majority of the measurements are carried out on both MEM-FLIM cameras. Results in the forms of figures and calculations on the MEM-FLIM2 camera are presented as an example, since the MEM-FLIM2 camera performs better than the MEM-FLIM1 camera. MEM-FLIM cameras are used to replace the conventional CCD camera and the image intensifier in the FD FLIM system. The fluorescence lifetime measurements using the upgraded FLIM system are also presented and discussed in this chapter.

This chapter is based upon and extended from the publication in the Journal of Biomedical Optics 17(12), 126020 (2012).

Keywords: FLIM, all-solid-state camera, pixel modulation, camera evaluation and comparison

8.1 Introduction

In chapter 6, we discussed two different architectures of MEM-FLIM cameras: transferring the charge to registers located in the horizontal direction at the modulation frequency (MEM-FLIM1) and transferring the photo-generated charge alternately to two adjacent CCD storage registers in the vertical direction (MEM-FLIM2). The architecture of the MEM-FLIM1 sensor is similar to an interline CCD, while MEM-FLIM2 to a full frame CCD. The advantage of MEM-FLIM1 design over the MEM-FLIM2 is that in the MEM-FLIM2 design the light source needs to be switched off during the image transfer period since the photogate of the sensor is also used for charge transfer. In the horizontal design, however, dedicated registers are used to transfer the charge, which means there will be no smear effect if the light is left on during image transfer. This disadvantage of MEM-FLIM2 design can be overcome by using a properly designed switchable light source. Evaluation results for both cameras are presented and discussed in the rest of this chapter.

8.2 System configuration and materials

8.2.1 System configuration

Our reference FLIM system includes an Olympus inverted microscope system IX-71 (Olympus), a LIFA system (Lambert Instruments, Roden, The Netherlands) which includes a LI²CAM Intensified CCD camera (GenII with S25 photocathode) as the reference camera (Lambert Instruments, Roden, The Netherlands) and a Dell computer installed with the Windows XP operating system. The MEM-FLIM system replaces the reference LI²CAM camera with our MEM-FLIM camera, while the rest of the system remains the same.

A 472 ± 15 nm single-band excitation filter (Semrock FF01-472/30-25, Rochester, U.S.A.), a 495 nm LP dichroic mirror (Semrock FF495-Di02-25 $\times$ 36) and a 520 ± 18 nm single-band emission filter (Semrock FF01-520/35-25) are used in the GFP (Green Fluorescent Protein) fluorescence filter cube. A 472 ± 15 nm single-band excitation filter (Semrock FF02-438/24-25, Rochester, U.S.A.), a 495 nm LP dichroic mirror (Semrock FF495-Di02-25 $\times$ 36) and a 520 ± 18 nm single-band emission filter (Semrock FF01-483/32-25) are used in the CFP (Cyan Fluorescent Protein) fluorescence filter cube for the Förster resonance energy transfer (FRET) experiment. An Olympus oil objective with a magnification of $100\times$ and an NA = 0.6 is used in the resolution measurement. A Zeiss air objective with a magnification of $20\times$ and a numerical aperture NA = 0.5, and a Zeiss oil objective with a magnification of $40\times$ and an NA = 1.3 are used in the lifetime measurements.

A 12V/100W halogen bulb (Eiko, model EVA) is used as a light source for characterizing the cameras. A LED (LUXEON Rebel, LXML-PR01-0225), the peak wavelength of which is at 460 nm, can be controlled (modulated) both by the reference FLIM system and the MEM-FLIM system and used for the lifetime measurements. The MEM-FLIM camera has a pixel size at $17 \mu\text{m}$ by $17 \mu\text{m}$. The reference system has an effective pixel

size at $20.6 \mu\text{m}$ by $20.6 \mu\text{m}$. A stage micrometer (Coherent 11-7796, U.S.A.) is used for measuring the sampling density of the cameras. An oscilloscope (LeCroy WAVE8URFER 64Xs) is used to monitor the waveform from the MEM-FLIM cameras. Agilent (81110A) pulse pattern generator is used to test the LED driven signal from the camera and the toggle gate waveform.

8.2.2 Materials

In order to determine the phase change and the modulation change introduced by the system itself, the system has to be calibrated with a fluorescent material with a known lifetime before carrying out subsequent lifetime experiments. We have used a $10 \mu\text{M}$ fluorescein solution (Sigma Aldrich 46955) ($\tau = 4 \text{ ns}$) [136, 137] for the system calibration. The fluorescein is dissolved in 0.1 M Tris buffer and the pH is adjusted to 10 using NaOH.

When testing the lifetime system performance, green and yellow fluorescent plastic test slides (Chroma, U.S.A) are often used as fluorescent samples in order to avoid photobleaching either a biological sample or a fluorophore solution. Fixed U2OS (osteosarcoma) cells that express GFP supplied from Leiden University Medical Center), and GFP-Actin labeling live cells (provided from the Netherlands Cancer Institute) were used for the fluorescent lifetime measurements.

The FRET sensor “mTurquoise-Epac-Venus-Venus” [138] was supplied by the Netherlands Cancer Institute. The donor in the FRET sensor, mTurquoise, is a novel, very bright and single-exponentially decaying CFP variant. By adding $1 \mu\text{l}$ IBMX (100mM) solution and $1 \mu\text{l}$ Forskolin (25mM) solution, the second messenger cyclic adenosine monophosphate (cAMP) is elevated. The FRET sensor undergoes a large conformational change when responding to cAMP change and the donor and the acceptor are physically separated. This results in a robust decrease in FRET which can be indicated by the increase of the fluorescence lifetime of the donor mTurquoise.

8.3 Camera characteristic - Performance

8.3.1 Linearity

A linear regression line is fit to the intensity data for various exposure times, as shown in Fig. 8.1. The MEM-FLIM2 camera exhibits linear photometric response for almost the entire dynamic range, resulting in the coefficient of regression > 0.999995 . Since one image consists of two phase images (“phase one” image and “phase two” image), we split these two phase images and analyze them separately. The offset of the linear fit is caused by the readout noise, which will be explained in Section 8.3.4.3.

8.3.2 Sampling density

As shown in Fig. 8.2 (a) and (b), in both the horizontal and vertical directions, the sampling densities of the MEM-FLIM2 camera are the same: $212 \text{ pixels} / 170 \mu\text{m} \approx 1.24$

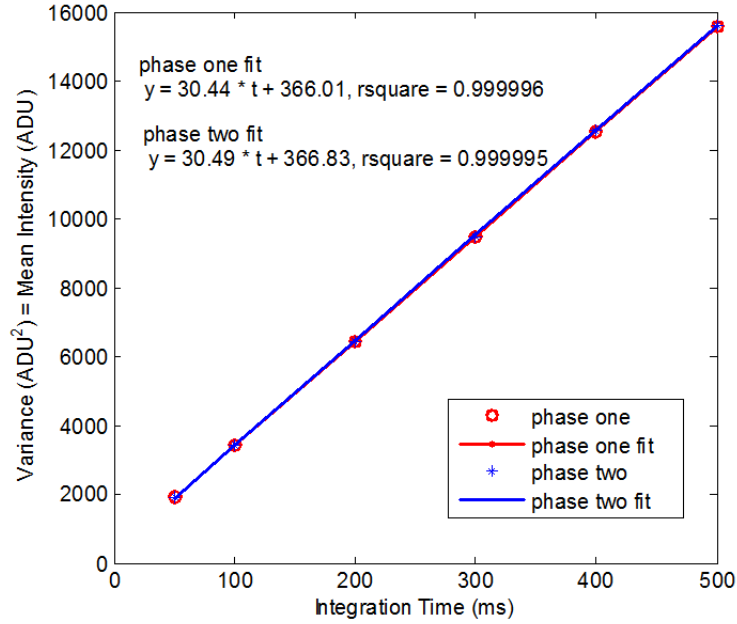


Figure 8.1: The linear photometric response of the MEM-FLIM camera.

samples per micron. $170 \mu\text{m}$ corresponds to the actual dimension of the section in the stage micrometer that is scanned (Fig. 8.2). The MEM-FLIM2 camera has a square sampling. The sampling distances are $170 \mu\text{m} / 212 \approx 0.8 \mu\text{m} = 800 \text{ nm}$. When dividing the pixel size ($17 \mu\text{m}$) by the magnification of the objective lens (20), we get $0.85 \mu\text{m}/\text{sample} \approx 1.18 \text{ samples}/\mu\text{m}$. This value differs from the measured sampling density ($1.24 \text{ samples}/\mu\text{m}$) due to internal demagnification in the microscope. The internal demagnification in the light paths of the MEM-FLIM systems and the reference system are different since the light paths of the two systems are not exactly the same.

Both the pixel size and the pixel number in the MEM-FLIM cameras are the same in the horizontal and vertical directions, however, the image has a rectangle shape. This is due to every image containing two phase images. If we assign the green color to one thresholded phase image and the red color to the other thresholded phase image, by overlapping the two phase images, we see that these two phases images match very well and result in the yellow color shown in Fig. 8.2 (c) and (d). Less than 2% of the pixels, as shown in Fig. 8.2, differ between the two thresholded phase images. The images of Fig. 8.2 (a) and (b) appear stretched due to two square image pixels in the vertical direction correspond to a single square pixel on the sensor with two storage areas.

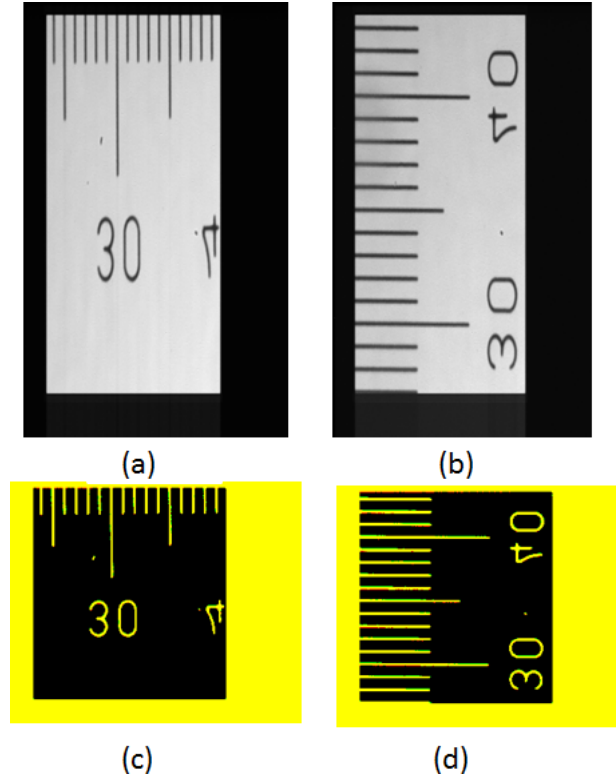


Figure 8.2: Illustration of using a stage micrometer to measure the sampling density. (a) Horizontal direction view, (b) vertical direction view, (c) the overlapping image of two phase images in (a), and (d) the overlapping image of the two phase images in (b).

8.3.3 Resolution

The comparison of the OTF of MEM-FLIM2 and the reference camera is shown in Fig. 8.3. The use of the stage micrometer (as in Fig. 8.2) with the knowledge of the actual CCD pixel size makes it possible to determine the absolute physical frequency of cycles/mm shown in Fig. 8.3. The effect of differing optical magnification between the two systems is thereby compensated. The OTF of the MEM-FLIM2 camera is higher than that of the reference camera. As a consequence, the image quality for the MEM-FLIM2 camera is better than for the reference camera. Actual images will be shown later. The (incoherent) diffraction cutoff frequency of the lens [139] is $f_c = 2NA/\lambda$ which for green light ($\lambda \approx 0.5\mu\text{m}$) and $NA = 0.6$ gives $f_c \approx 2400\text{cycles/mm}$. The limiting factor in the OTF above is, therefore, not the objective lens but the camera system. The slight increase of the MEM-FLIM OTF above the objective lens OTF has two sources. First, all three curves have been normalized to unity although the exact transmission at $f = 0$ for the two cameras is probably less than one, and second, there is a slight amount of partial coherence associated with the condensor optics.

Besides comparing MEM-FLIM2 and the reference camera, a Hamamatsu camera

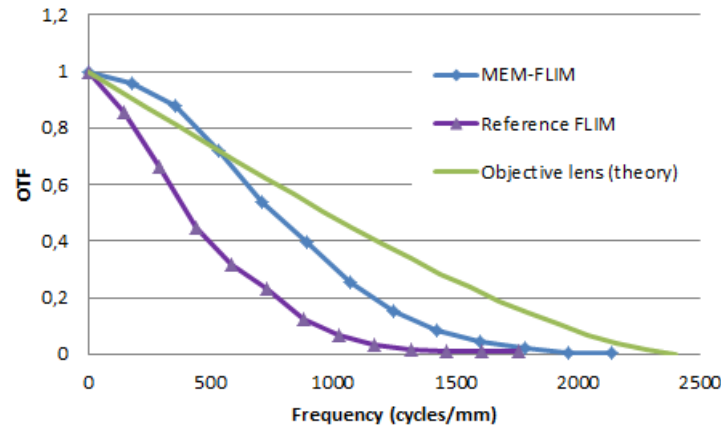


Figure 8.3: OTF comparison between the MEM-FLIM2 system, the reference FLIM system and the diffraction-limited objective lens.

(Hamamatsu Photonics, model C4742-80-12AG) and a Sony camera (Sony, XC-77) are also used for comparison. The pixel size of the Hamamatsu and Sony cameras are 17 and $6.45 \mu\text{m}$, respectively. Among these four cameras, only the reference camera employs an image intensifier. Figure 8.4 shows that the performance of the MEM-FLIM2 camera is comparable with the other two all-solid-state cameras, while the reference camera has a poorer performance due to the image intensifier. The influence of the wavelength on the

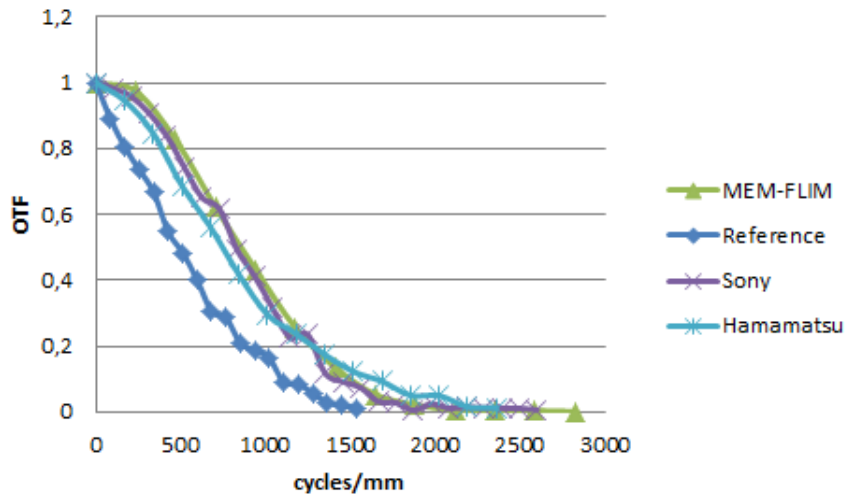


Figure 8.4: OTF comparison between four different cameras.

resolution is also investigated by inserting a red or green filter in the light path from the halogen light source to the camera. The peaks of the wavelengths received by the camera

without filter in the light path is 669 nm, and the wavelength peaks after inserting a red or a green filter are 670.4 and 554.0 nm. As shown in Fig. 8.5, the shorter the wavelength is, the better resolution is (indicated by the higher OTF). This result is consistent with the relationship of the wavelength and the resolution discussed in Eq. (2.4) and Eq. (2.5).

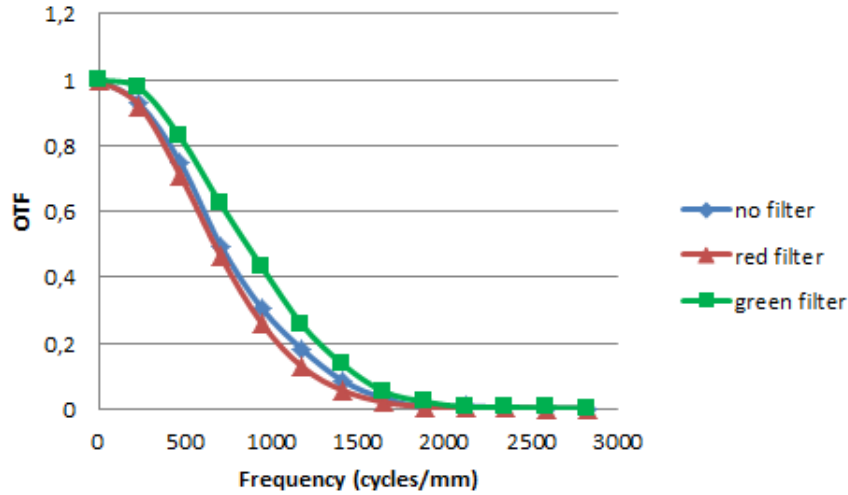


Figure 8.5: OTF comparison between different wavelengths.

8.3.4 Noise

8.3.4.1 Poisson noise distribution

The validation of the Poisson distribution model of the noise source is shown in Fig. 8.6. The linear fit indicates that the variance of the difference images increases linearly with the mean intensity, which shows that the noise source in the image is Poisson distributed. The integration time is 180 ms.

8.3.4.2 Dark current noise

Figure 8.7(a) shows the relationship between dark current and integration time when the frame time is fixed for the MEM-FLIM2 camera. The mean value of each column in a dark image is calculated and plotted for different integration times. By subtracting two images obtained at the same setting, the offset and the fixed pattern of each image can be eliminated. Since dark current noise follows Poisson statistics, the variance in this difference image equals twice the average intensity in one image [135]. The generated dark current is linear in the integration time, which is plotted in Fig. 8.7(b). When the integration time is 600 ms, the dark current is $76/16383 \approx 0.3\%$ of the full dynamic

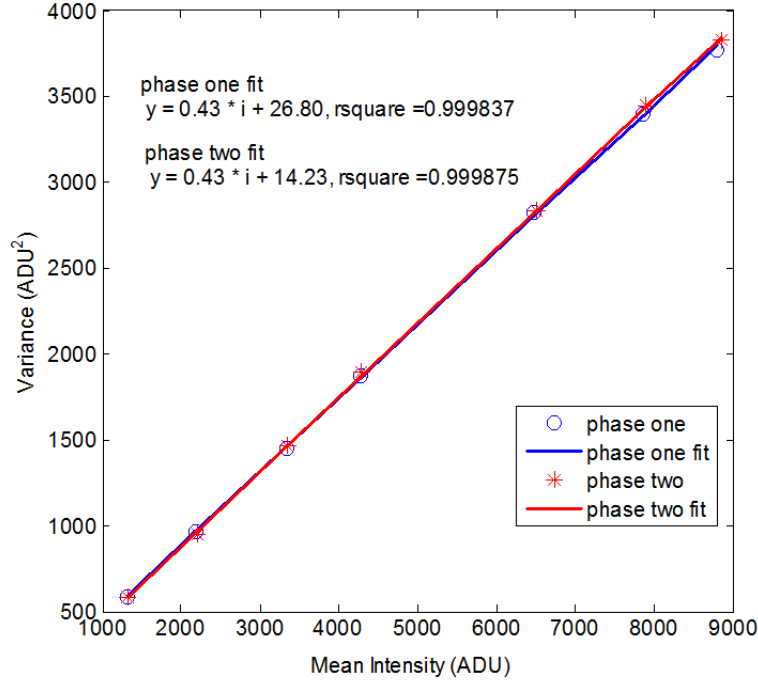


Figure 8.6: The Poisson assumption validation and the sensitivity of the MEM-FLIM2 camera.

range. Since the electron to ADU converting factor is known from the absolute sensitivity experiment, which is 0.43 ADU/e^- , the dark current can also be written as $76 \text{ (ADU)}/0.43 \text{ (ADU/e}^-)/600 \text{ (ms)} = 0.29 \text{ e}^-/\text{ms}$. By fixing the integration time and varying the frame time, we see in Fig. 8.8 that the dark current is not influenced by the frame time and can be neglected.

8.3.4.3 Readout noise

Readout noise can be obtained from the fittings in Fig. 8.7(a). When the integration time goes to zero, the noise source due to the dark current is eliminated. Thus the constant terms in the fittings represent the readout noise. The readout noise is independent of the integration time. The average readout noise of the MEM-FLIM2 camera is $\sigma_{\text{readout}} = \sqrt{((34.76 + 34.58)/2)} \approx 5.9 \text{ ADU} \approx 14 \text{ e}^-$. In the same way, the readout noise from the reference system can be determined to be $3.4 \text{ ADU} \approx 6 \text{ e}^-$ (figure not shown). The factor of 1.7 between these two results is most likely due to the fact that we are working with the first version of the MEM-FLIM chip/camera while the reference system, as an existing commercial product, is already well optimized.

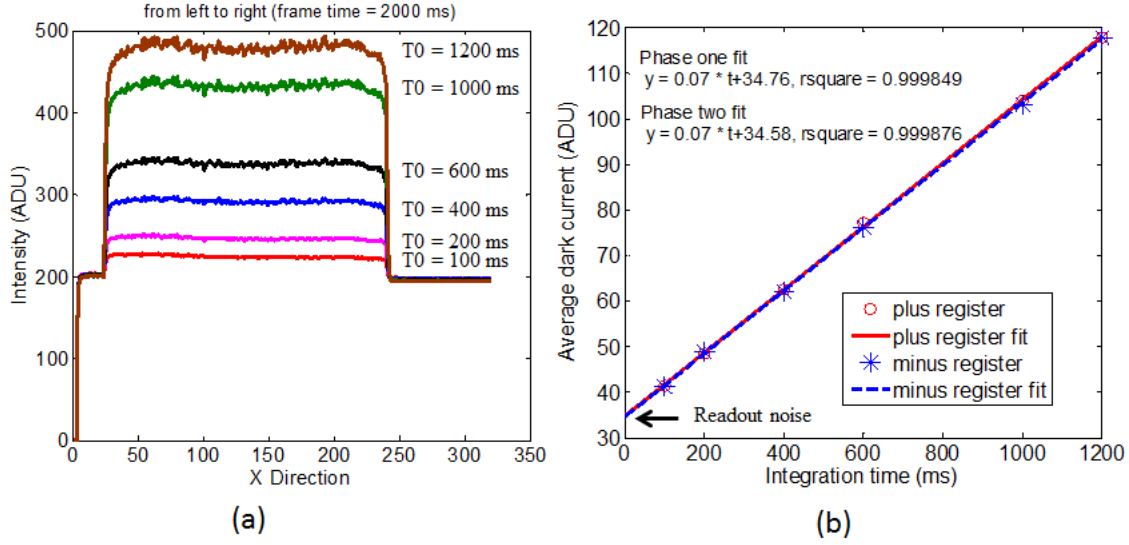


Figure 8.7: Dark current derived from the fixed frame time of 2000 ms. (a) The relationship between dark current and integration time (T_0), and (b) linearity of dark current.

8.3.5 Sensitivity

8.3.5.1 Sensitivity

The sensitivity of the MEM-FLIM2 camera is shown in Fig. 8.6. The linear fit indicates the noise source in the image is Poisson distributed, as explained in Section 8.3.4.1, and the slope of the fitting represents the sensitivity of the camera (Eq. (7.8)). There is a uniform sensitivity response across the sensor. The differences between the sensitivities of different regions for the MEM-FLIM2 cameras are quite small, as shown in Table 8.1. The sensitivity of the MEM-FLIM2 camera is 0.43 ± 0.03 ADU/e⁻. For the reference camera the same procedure resulted in a sensitivity of 0.53 ± 0.03 ADU/e⁻. For these experiments, the analog gain of the MEM-FLIM camera was set to 6 dB, and the MCP voltage of the reference camera was set to 400 V.

8.3.5.2 Detection limit

We can determine the minimum signal that can be detected by the MEM-FLIM2 camera from Eq. (7.9). When the integration time is short, the noise floor σ_n will be dominated by the readout noise σ_r . From Fig. 8.7(b) and Fig. 8.6, we know that $\sigma_n = \sigma_r = 5.9$ ADU ≈ 5.9 (ADU)/0.43 (ADU/e⁻) = 13.72 e⁻. We assume that the signal can be distinguished from the noise floor if the difference between the noise floor and the signal is k times bigger than the standard deviation of the signal: $\mu_s - \sigma_n \geq k\sigma_s$ (Eq. (7.9)). When $k = 5$, based upon the Chebyshev Inequality [140] the probability that the signal level can be mistakenly identified as noise will be $\leq 1/k^2 = 4\%$. The Chebyshev

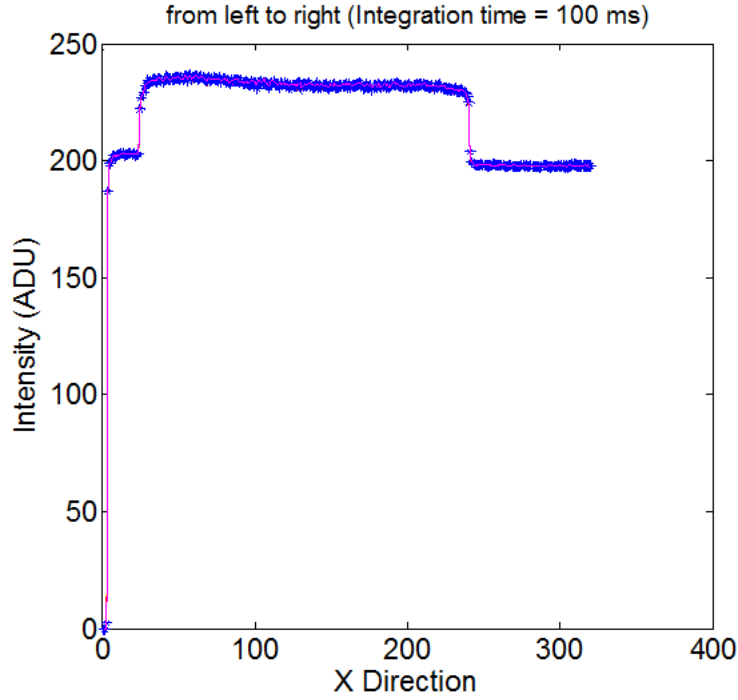


Figure 8.8: The relationship between dark current and frame time (T1) when the integration time is fixed to 100ms. The frame time is set from 200 ms up to 2000 ms in intervals of 200 ms. The results from different frame time values are overlapped with each other.

Inequality is distribution free so it is not necessary to know the probability distribution of the signal. If we make use of the assumption that the signal has a Poisson distribution and that the average value of the signal is sufficiently high ($\mu_s > 10$), then the probability given above drops to 3×10^{-6} . This means signal detection at the $k = 5$ level is essentially guaranteed. In this case using Eq. (7.9) the minimum signal that can be detected by the MEM-FLIM2 camera is $\mu_s = 48.6 \text{ e}^-$. Using the same method, the minimum signal that can be detected by the reference camera is 35.4 e^- .

8.4 Lifetime measurement

We have measured the fluorescence lifetime of various objects, e.g. fluorescent solution, and biological samples. Below are examples of the lifetime measurements on biology samples: fixed U2OS (osteosarcoma) cells that expressed GFP supplied from Leiden University Medical Center, GFP - Actin labeling live Hela cells, and GFP - H2A labeling live U2OS cells provided from The Netherlands Cancer Institute. In all experiments, the calibration is done to determine the phase and modulation change introduced by the system itself by using a fluorescein solution at $10 \mu\text{M}$, the lifetime of which is known to be 4ns [136, 137]. The modulation frequency of the MEM-FLIM2 system is at this time hard-

Table 8.1: Sensitivities of different regions of the MEM-FLIM2 camera.

Region name	Pixel number	Sensitivity
Middle	[100:110, 100:110]	0.4112
Upper left	[10:20, 1:11]	0.4556
Upper right	[10:20, 195:205]	0.3931
Lower left	[195:205, 1:11]	0.4566
Lower right	[195:205, 195:205]	0.4173
Middle left	[100:110, 1:11]	0.4434
Middle right	[100:110, 195:205]	0.4617
Upper middle	[10:20, 100:110]	0.4115
Lower Middle	[195:205, 100:110]	0.4017
Average		0.4280
Stdev		0.0263

wired in the MEM-FLIM2 camera to 25 MHz. Results from the reference system served as a basis for comparison. The typical fluorescence lifetime of GFP is 2-3 ns [122, 141].

8.4.1 GFP labeling fixed U2OS cells

The comparative lifetime measurement was performed on the fixed GFP cell shown in Fig. 8.9. U2OS is a human osteosarcoma cell line. A Zeiss objective with a magnification of 20 $\times$ and a numerical aperture of 0.5 was used for this experiment. The integration time of the camera system for the sample in both systems was set to 100 ms.

In order to compare images from two cameras, the histograms of the two images are stretched over the range of 0 to $2^{BN} - 1$. One maps the intensity value $p_{low}\%$ to the value 0 and $p_{high}\%$ to $2^{BN} - 1$ by the transformation given in Eq. (8.1) [142]. The original intensity A at position $[x,y]$ then transforms to B . In our case, we choose $p_{low}\%$ and $p_{high}\%$ to be 5% and 99.9% to exclude the outliers. BN is chosen to be 8, so the mapped intensity range is from 0 to 255. Note that the values of $B[x,y]$ are floating point numbers.

$$B[x, y] = \begin{cases} 0 & A[x, y] \leq p_{low}\% \\ (2^{BN} - 1) \cdot \frac{A[x, y] - p_{low}\%}{p_{high}\% - p_{low}\%} & p_{low}\% < A[x, y] < p_{high}\% \\ (2^{BN} - 1) & A[x, y] \geq p_{high}\% \end{cases} \quad (8.1)$$

We can see that the field of view of the reference camera is bigger than the MEM-FLIM2 camera in Fig. 8.9(a) and (c), but the resolution of the MEM-FLIM2 camera is significantly better than the reference camera in Fig. 8.9(b) and (d). Detailed structure

inside the cell can be seen on the image, which is taken with the MEM-FLIM2 camera. This structure is not readily visible in the image with the reference camera.

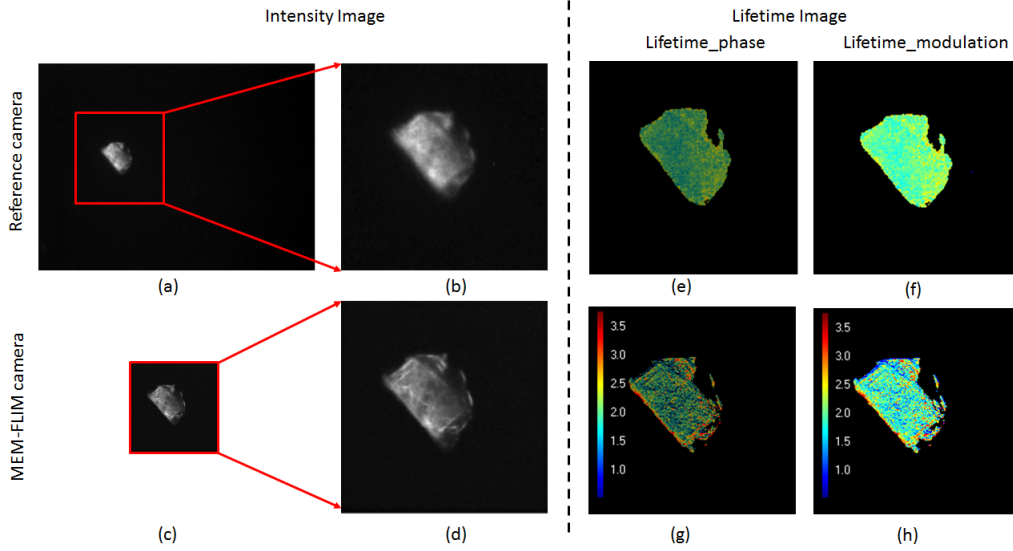


Figure 8.9: Intensity and lifetime images of fixed U2OS GFP cells. (a-d) are intensity images and (e-h) are lifetime images. (a) The intensity image from the reference camera, (b) the magnified image of (a), (c) the intensity image from the MEM-FLIM2 camera, (d) the magnified image of (c), (e) the lifetime derived from the phase change for the reference camera, (f) the lifetime derived from the modulation depth change for the reference camera, (g) the lifetime derived from the phase change for the MEM-FLIM2 camera, and (h) the lifetime derived from the modulation depth change for the MEM-FLIM2 camera

The lifetime images from the both cameras are compared in Fig. 8.9 (e-h). The MEM-FLIM2 camera clearly yields a better spatial resolution in the lifetime images. A 10×10 pixel area was used corresponding to an area of $87 \mu\text{m}^2$ for the reference camera and $65 \mu\text{m}^2$ for the MEM-FLIM2 camera. The measurement results are shown in Table 8.2. The lifetime uncertainty is the standard deviation of the 100 lifetimes in the 10×10 pixel area. The difference between the lifetimes derived from the phase change and the modulation change can be explained by the heterogeneity of GFP lifetime components. By doing multi-frequency measurements on the reference system, the lifetime components in the sample are determined to be 1.24 ns (41%) and 5.00 ns (59%). The data are consistent with the values in the literature (1.34 ns (46%) and 4.35 ns (54%)) [143].

The fluorescent lifetime, as recorded with the MEM-FLIM2 camera, is in good agreement with values from the reference camera. Compared to the reference camera, the lifetime uncertainties ($\sigma's$) measured from the MEM-FLIM2 cameras are higher than those from the reference camera since the modulation depth for the MEM-FLIM camera is not (yet) as good as in the reference camera. One possible reason for the lower modulation depth for the MEM-FLIM camera is the mask displacement, which will be explained in

Table 8.2: The lifetime results of GFP labeling fixed U2OS cells.

	Reference camera	MEM-FLIM camera
τ_θ (ns)	1.96 ± 0.31	1.86 ± 0.48
τ_m (ns)	3.05 ± 0.21	3.20 ± 0.58
modulation	0.64	0.55

8.5.5. However, image quality (detail) of the MEM-FLIM2 camera is significantly better than that of the reference system.

8.4.2 GFP - Actin labeling HeLa cells

For these experiments we imaged HeLa cells, stably expressing GFP-tagged β -actin with the MEM-FLIM2 and reference cameras. The β -actin expression in these cells is quite low and they therefore present an example of a typical low-intensity preparation. A Zeiss oil objective with a magnification of $40\times$ and a numerical aperture of 1.3 was used for this experiment. The integration time for both the reference camera and the MEM-FLIM2 camera was 1000 ms. The same gray value stretching processes as described in Section. 8.4.1 were applied to the intensity images.

The results of the lifetime measurements are shown in Tab 8.3. The lifetimes derived from the phase change for the reference camera and the MEM-FLIM2 camera are 2.66 ± 0.49 ns and 2.59 ± 0.40 ns, and the lifetime derived from the modulation depth change are 2.35 ± 0.97 ns and 2.63 ± 1.46 ns, respectively. The modulation on the sample for the reference system reached 1.05 while the value for the MEM-FLIM2 camera was 0.38. From Fig. 8.10, we can see that the MEM-FLIM2 camera has a higher resolution and a better image quality than the reference camera. The fibers in the cell can be seen in the MEM-FLIM2 image but not in the reference image.

The lifetime images derived from the phase change of both cameras are also compared in Fig. 8.10 (d-f). In the lifetime image of the MEM-FLIM2 camera, the difference within the cell -the spatial variation- can be seen. Just above the middle of the image the lifetime (color) differs from the surrounding cellular material (as shown within the white rectangle). This structure can also be seen in the intensity image. This detail is blurred in the lifetime image from the reference camera.

8.4.3 GFP - H2A labeling live U2OS cells

For these experiments we imaged U2OS cells, stably expressing GFP-H2A with the MEM-FLIM2 and reference cameras. A Zeiss oil objective with a magnification of $40\times$ and a numerical aperture of 1.3 was used for this experiment. The image comparison in Fig. 8.11 again shows that the MEM-FLIM2 camera has a higher resolution than the

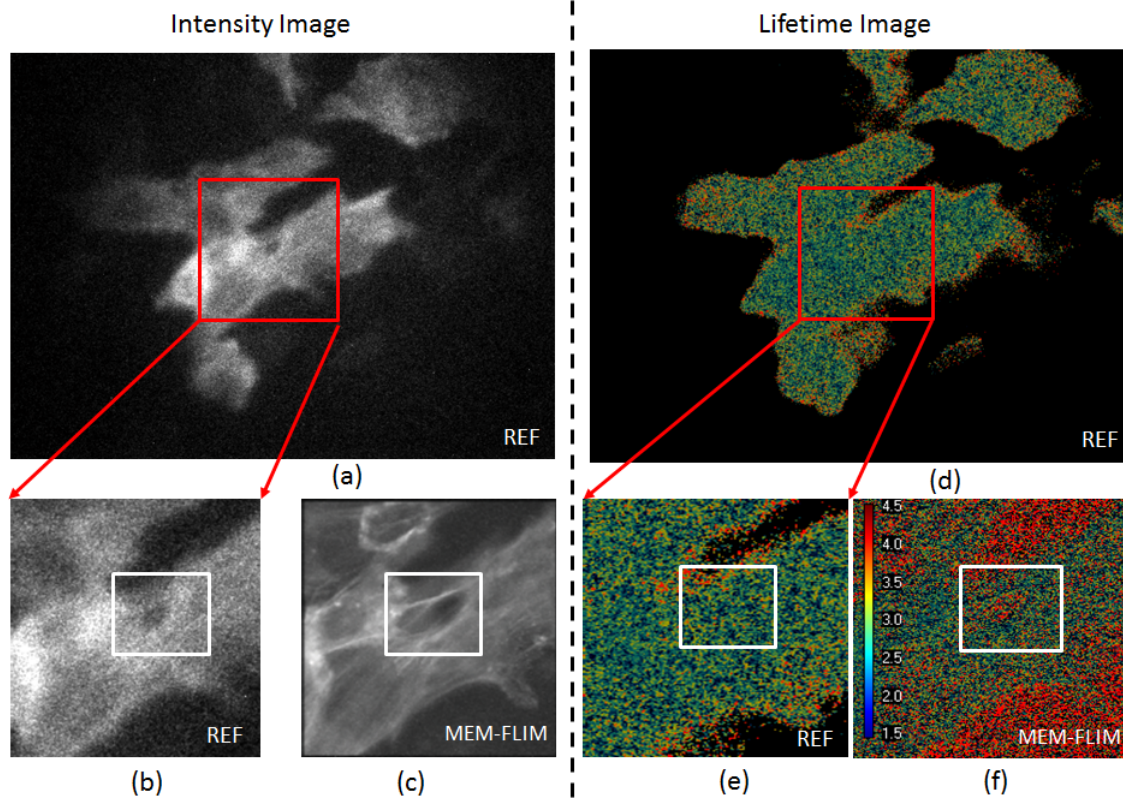


Figure 8.10: Intensity and lifetime images of GFP-Actin labeling HeLa cells. (a-c) are intensity images and (d-f) are lifetime images (the lifetime derived from the phase change): (a,d) the full field of view from the reference camera, (b,e) a magnified region from the reference camera, and (c,f) the same region from the MEM-FLIM2 camera.

reference camera, while the reference camera has a larger field of view than the MEM-FLIM2 camera. The integration time for both the reference camera and the MEM-FLIM2 camera was 200 ms, and the phase-based, lifetime results are comparable with 2.65 ± 0.48 ns measured by the MEM-FLIM2 camera and 2.57 ± 0.20 ns measured by the reference system. The same gray value stretching processes as described in Section. 8.4.1 were applied to the intensity images.

8.4.4 Förster resonance energy transfer experiment

For these experiments we monitored the donor lifetime change of the FRET sensor with the MEM-FLIM2 camera. Time-lapse experiments were carried out. The integration time of each phase was 150 ms, and lifetime experiments were carried out every 210 ms. A movie of fluorescence lifetime change can be made from the time-lapse experiments, in this case, at 4.8 frame per second. The whole experiment lasted for 177 s. The intensity and lifetime images are shown in Fig. 8.12. The phase-based lifetime increased from 2.98

Table 8.3: The lifetime results of GFP labeling Actin labeling HeLa cells.

	Reference camera	MEM-FLIM camera
τ_θ (ns)	2.66 ± 0.49	2.59 ± 0.40
τ_m (ns)	2.35 ± 0.97	2.63 ± 1.46
modulation	1.05	0.38

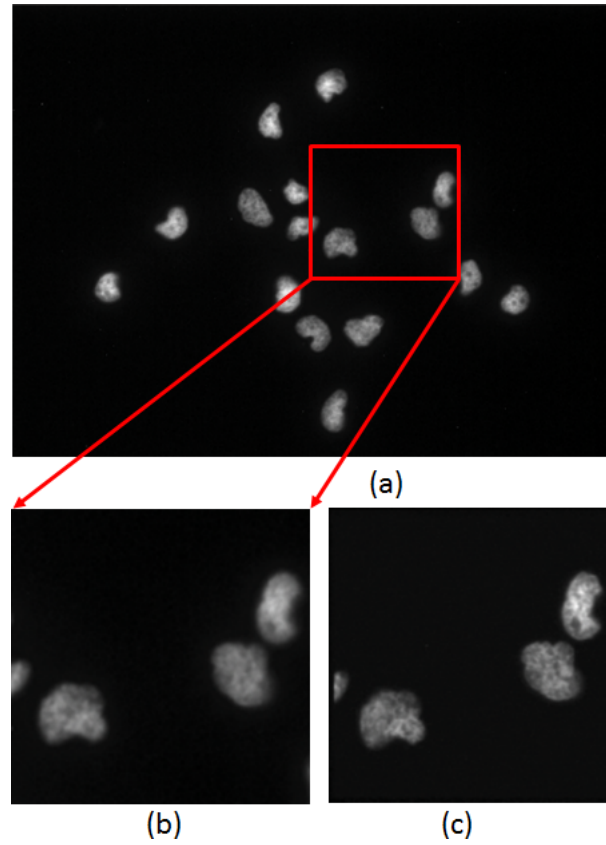


Figure 8.11: Intensity images of live U2OS cells, (a) the full field of view from the reference camera, (b) a magnified image of a region from the reference camera, and (c) the same region from the MEM-FLIM2 camera.

± 0.39 ns to $3.55 \pm$ ns. The lifetime increased by 19%.

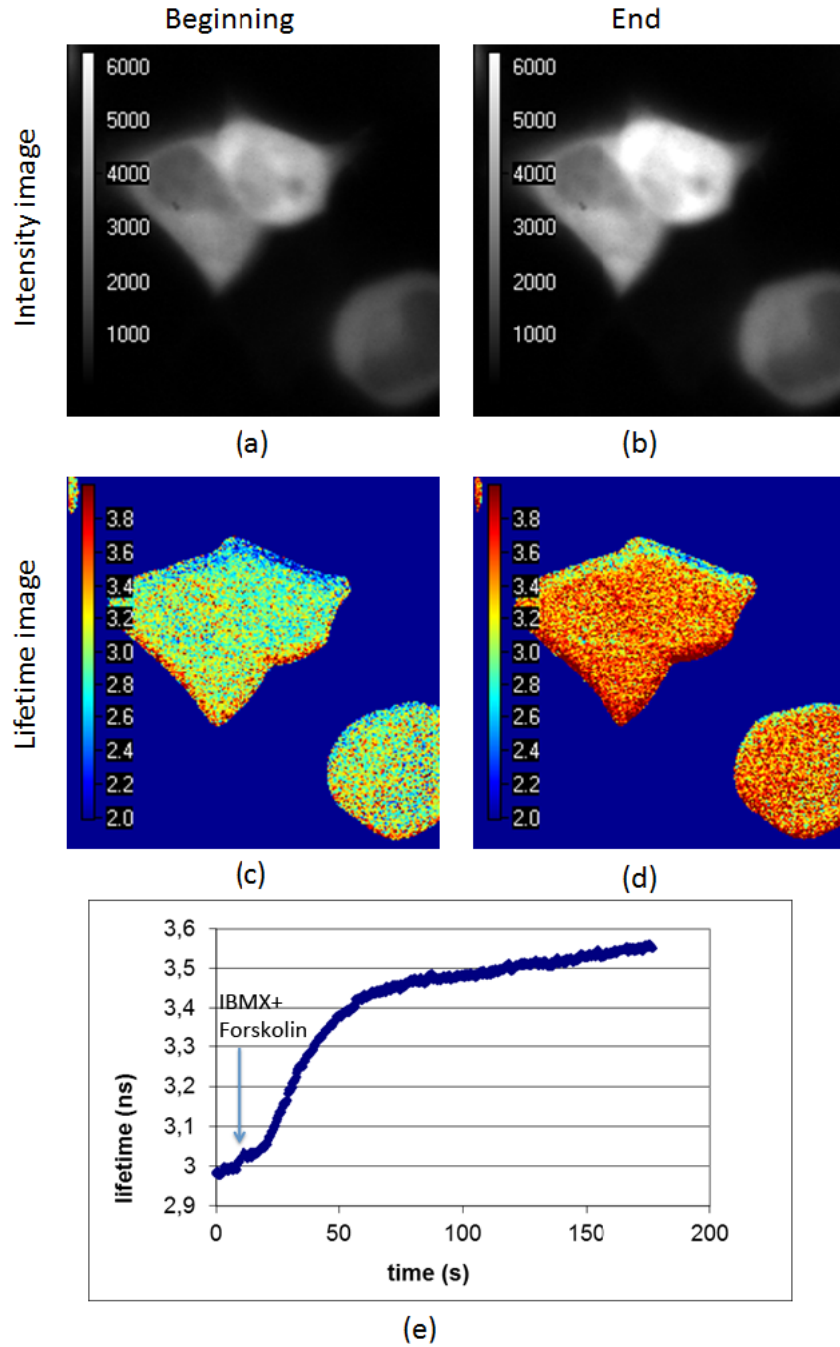


Figure 8.12: Fluorescence lifetime change of FRET experiment: (a) and (b) are intensity images at the beginning and the end of the experiment, (c) and (d) are fluorescence lifetime images at the beginning and the end of the experiment, (e) the change of phase-based fluorescence lifetimes.

8.5 Imperfection of the MEM-FLIM cameras

8.5.1 Charge transfer efficiency

MEM-FLIM1 suffers from charge loss in the vertical register, as shown Fig. 8.13. A fluorescent test pattern from Edmund Optics (DA050E, Fluor USAF target 3×3

NEG) is used in this experiment. The illumination intensity from the LED light source is controlled by the LED driven current. The red box in Fig. 8.14(a) shows that at higher row numbers, there are remaining charges, which can also be observed at the area below the image pattern in Fig. 8.13. It looks like the pattern produces a tail below it. When the SNR is high at a high intensity illumination, this tail effect is not obvious, for example, in 8.13(a). At lower SNR circumstances, however, the charge transfer inefficiency not only causes a more obvious tail below the pattern, but also distorts the pattern shapes, as shown in Fig. 8.13(b-d). This tail effect is likely caused by the gate connection designs of the vertical gates. The low vertical charge transfer efficiency (0.935) makes MEM-FLIM1 unsuitable for the fluorescence lifetime measurements of biological samples. Most biological samples emit limited amount of photons and the acquired intensity image can be severely distorted by the charge transfer inefficiency, as shown in Fig. 8.15. Fixed U2OS (osteosarcoma) cells that expressed GFP supplied from Leiden University Medical Center were used in this experiment.

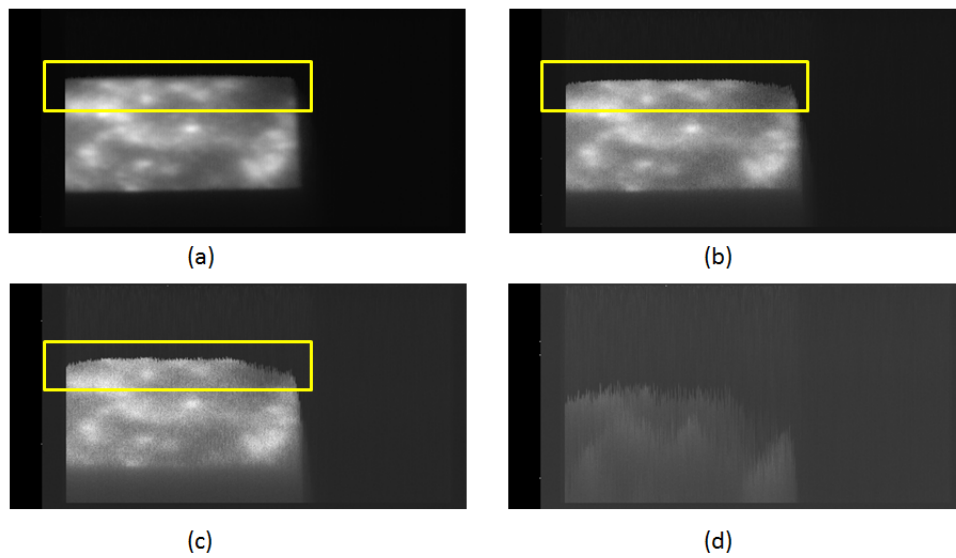


Figure 8.13: Charge transfer inefficiency effect on MEM-FLIM1 camera. The current input of the LED light is (a) 350 mA, (b) 100 mA, (c) 50 mA and (d) 5 mA.

MEM-FLIM2, on the contrary, has a much higher vertical charge transfer efficiency (0.999989) and outperforms the MEM-FLIM1. We focus, therefore, on the vertical toggling MEM-FLIM2 design as the architecture-of-choice for the system. The evaluation results above and following lifetime measurements are, therefore, based on the MEM-FLIM2 cameras.

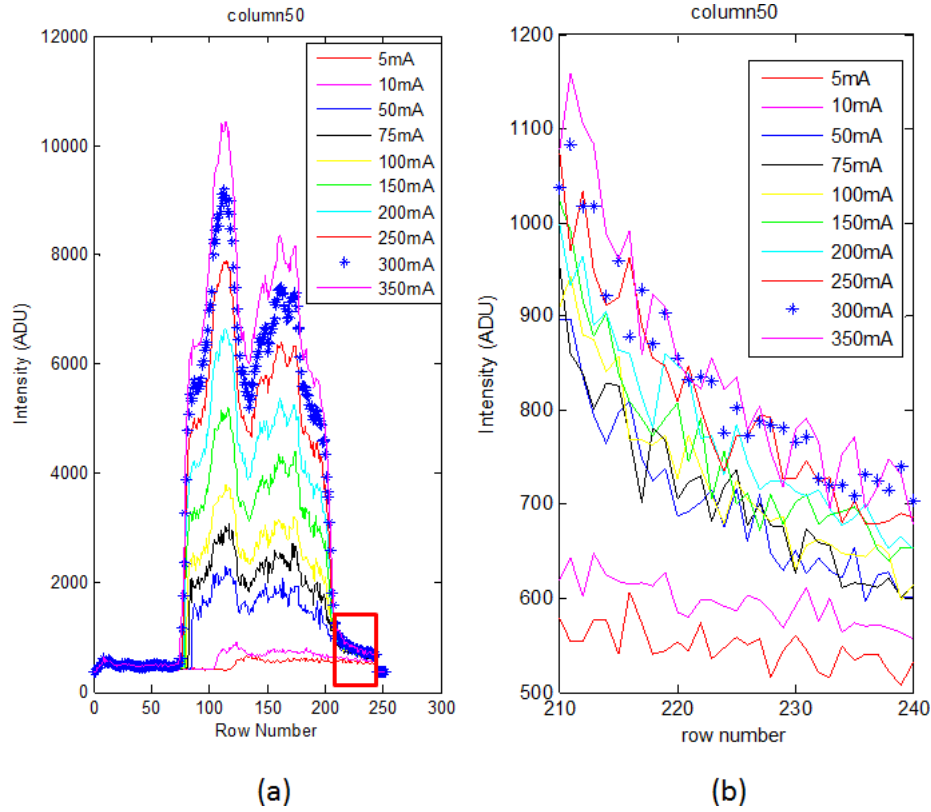


Figure 8.14: Tail effect due to the charge transfer inefficiency for MEM-FLIM1 camera. (a) Intensity plot of a column (column number 50) of Fig. 8.13, where the tail effect can be seen in the region marked with red outline. (b) A zoomed in plot for the red box region in (a).

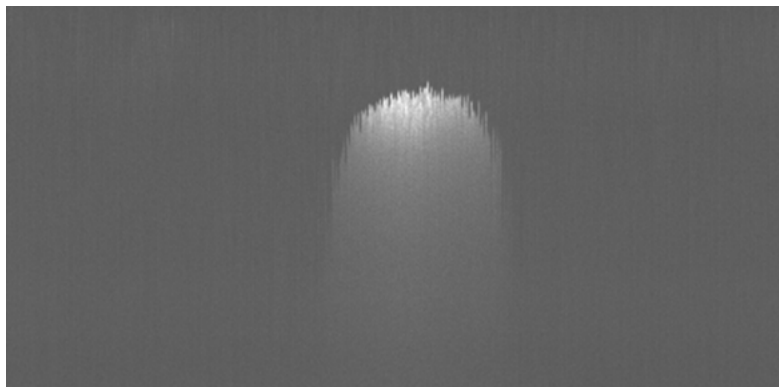


Figure 8.15: GFP cell image from MEM-FLIM1 camera.

8.5.2 Temperature

Temperature is one of the main factors that can influence the dark current generated by the CCD sensor. It is important that the temperature of the sensor remains stable

when the camera is operated. The temperatures of the MEM-FLIM2 sensor and camera are measured using a FLUKE (TI10) thermal imager, as shown in Fig. 8.16. We have noticed that the driver of the camera becomes quite hot when the camera is in operation, as shown in the red area in Fig. 8.16(b). The temperature can go up to 92°C at the driver chip. The sensor temperature remains at 34°C during the operation when the camera boards (including sensor chip) are not mounted in a camera housing. In order to mount the camera on the microscope, an aluminum housing with air circulation slots was made as shown in Fig. 8.16(c). The sensor temperature remains at 34°C inside the camera housing with a fan forcing the air to circulate in order to prevent heat accumulation inside of the housing. The air is sucked in through the filter layer in the fan to the camera boards, and comes out from the slots on the housing. The setup is shown in Fig. 8.16(c). Figure 8.16(d) shows the front view of a C mount of the camera housing, through which the sensor temperature can be measured.

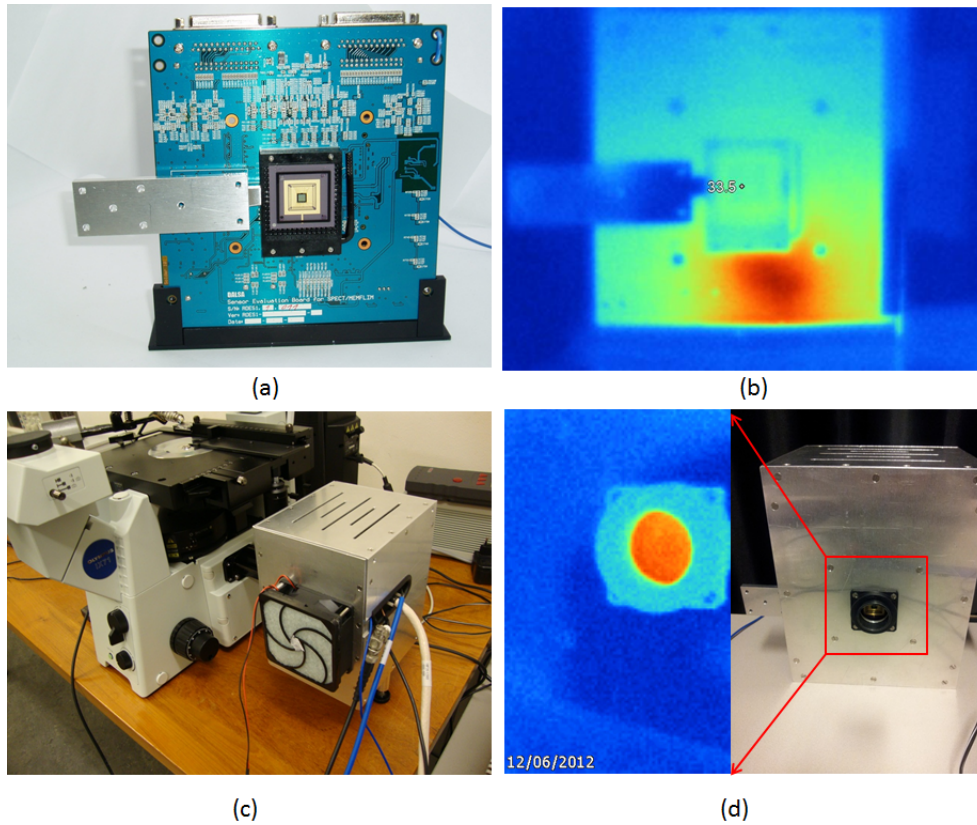


Figure 8.16: Temperatures of MEM-FLIM2 sensor and camera. (a) MEM-FLIM2 sensor and camera board, (b) the sensor temperature when the camera is in operation, (c) the MEM-FLIM2 aluminum housing mounted on the microscope, and (d) the front view of a C mount of the camera housing, through which the sensor temperature can be measured.

The forced air cooling is not the optimal way to cool down the sensor due to the

vibrations it might cause to the optical system. It is necessary, however, to keep the temperature down when the camera sits in the housing. We have noticed an undesired interference pattern on the dark current after switching on the camera for 10 min without forced air flowing, as shown in Fig. 8.17(a). Figure 8.17(b) shows the intensity plot versus row number, the intensity value of a specific row is a mean value of the intensities over the whole row. The integration time of this experiment is 100 ms. After switching on the fan and forcing the air to circulate, the interference pattern disappears and a uniform dark current image is generated. For this reason, all subsequent experiments with MEM-FLIM cameras were done with the fan on.

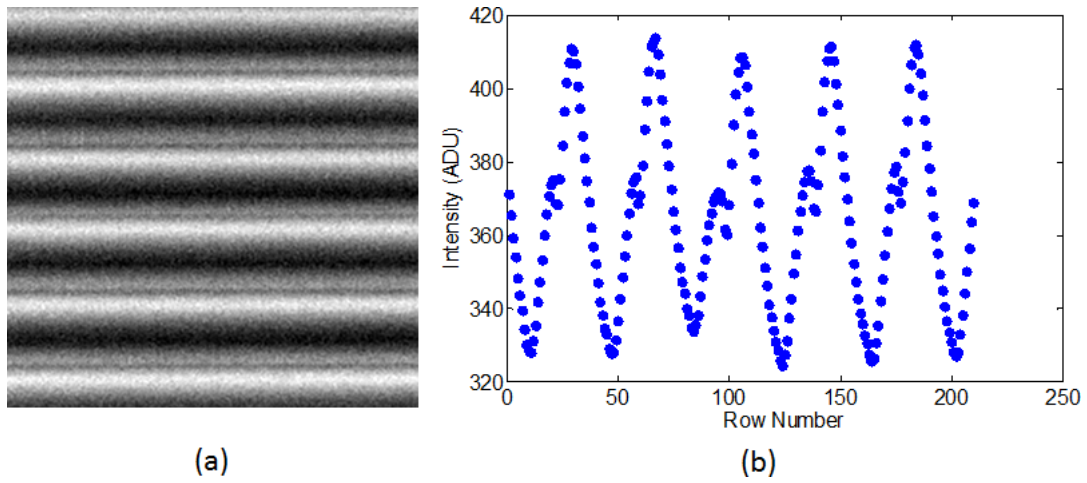


Figure 8.17: The interference dark current pattern without forced air cooling. (a) The dark image of MEM-FLIM2 without forced air cooling, and (b) the plot of the averaged row intensity from the top to the bottom.

8.5.3 Analog-to-digital converter

The Analog-to-digital converter (ADC) in a CCD camera is a device that converts an input analog voltage to a digital pixel value which represents the amplitude of the continuous signal. In the MEM-FLIM cameras, ADCs of 14 bits are used and in total we can have 0 to $2^{14} - 1 = 16383$ different levels of the intensity. We have found that the third lowest bit of the converter has a systematic error in encoding the signal. Figure 8.18(a) shows the image from which we spotted this phenomenon. The integration time of the camera is 180 ms. Due to the imperfection of the CTE in vertical direction, the region below the test pattern also shows faded bar patterns. We plot the histogram of the region highlighted in the yellow box from Fig. 8.18(a) in Fig. 8.18(b). The two peaks in the histogram indicate the light bar patterns in the dark region. The zoomed-in histogram between the two peaks, the region which is marked with red box in Fig. 8.18(b), is shown in Fig. 8.18(c). The plot in Fig. 8.18(c) shows a periodic pattern. The obvious lowest

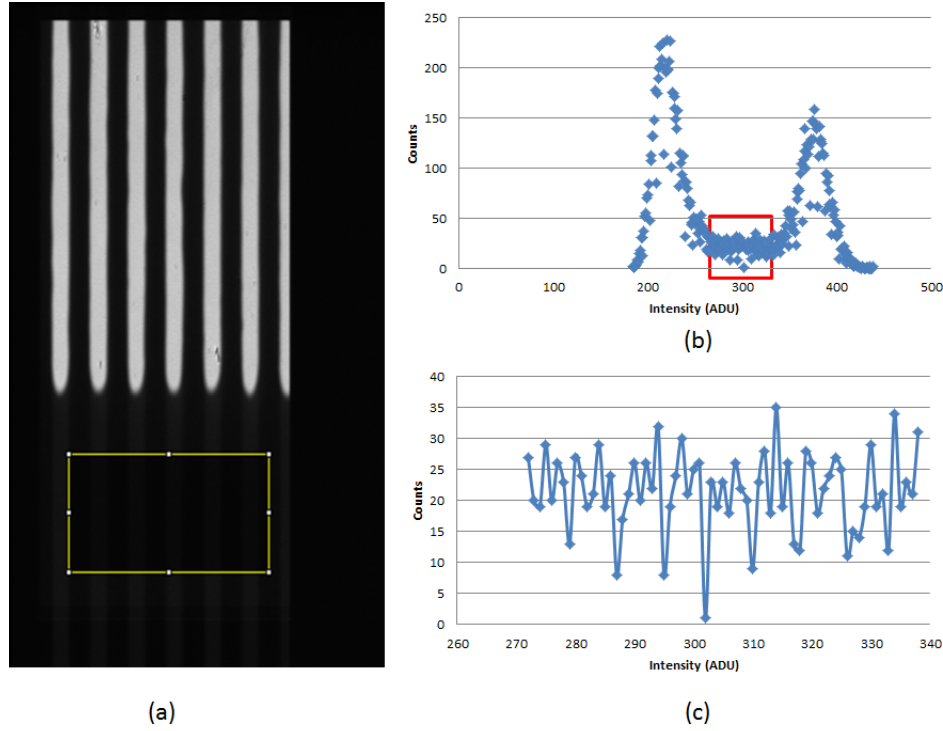


Figure 8.18: The ADC defect of the MEM-FLIM2 camera. (a) The test pattern used to spot the ADC defect, (b) the histogram of the yellow box region in (a), and (c) the zoomed in histogram of the red box region in (b).

value occurs at every $2^3 = 8^{th}$ intensity level, indicating the imperfect performance of the third lowest bit of the converter. The influence of this fluctuation compared to a 16383 level gray value image, however, is small enough to be currently ignored.

8.5.4 LED driven signal and toggle gate signal

In the MEM-FLIM system, the modulation signal for the LED comes from the MEM-FLIM camera. The modulation for the LED and the demodulation on the toggle gate on the pixel are at the same frequency. When doing fluorescence lifetime, delays are introduced between these two signals by changing the phase step. Intensity images at different phase steps are taken to extract the phase change and the modulation depth information. The phase delay of the LED driven signal can be changed at every 15 degrees, equivalent to 24 phases in one periodic cycle. The intensity curve at different phase steps should follow a sinusoidal wave, as explained in Chapter 3. When plotting the average intensities of the images at different phase steps from the MEM-FLIM2 camera (the delay signal for the light source is controlled by the MEM-FLIM2 camera), the intensity data are not uniformly displayed, as shown in red circles in Fig. 8.19(a). At certain phase steps, the two intensity data are either too far away apart or too close to

each other. If we use a modulation signal and control its phase delay from an external source (Agilent pulse pattern generator) with a set pulse width (20 ns), we found that this uneven distribution phenomenon disappears, which yields a more reasonable curve, as shown in Fig. 8.19(b). The phase delay of the LED driven signal generated by the Agilent pulse pattern generator is also set at every 15 degrees.

In order to find out the difference between using a driven signal for LED from the MEM-FLIM2 camera and the Agilent generator, we closely examined the MEM-FLIM2 camera output signal used for the phase delay in the previous experiment. While the pulse from the Agilent pulse pattern generator has a fixed width, both the width and pulse shape from the MEM-FLIM2 camera at different phase steps are varying, as shown in Fig. 8.20 and Fig. 8.21. The average width of the LED pulse is 19.2 ns with a standard deviation of 0.3 ns. The varied shape and width of the LED driven pulse causes the unevenly distributed intensity value over different phase steps showed in Fig. 8.19(a). Despite this variation, the LED driven signal is quite stable over a period of time, as shown in the persistence image in Fig. 8.22. In this case, the LED driven signals generated in 30 min are plotted on top of each other. The oscilloscope is triggered by the frame signal from the camera for all the waveforms monitored in this section at a frame time of 200 ms. The signal sampling rate is 2.5 GB/s.

To generate intensity images at different phase steps, the demodulation signal applied on the toggle gate is as important as the modulation signal for the LED. We have verified that changing the phase steps does not influence the signal shape and the width on the toggle gate. The waveform of the demodulation signal on the toggle gate and the camera output signal which drives the LED are shown in Fig. 8.23. The zoomed-in channel 3 (Z3: the blue curve at the bottom part of the figure) is the camera output signal which drives the LED. The zoomed-in channel 4 (Z4: the green curve at the bottom part of the figure) is the camera output signal which drives the LED. Thus we ruled out the influence of the toggle gate demodulation signal on the different results between Fig. 8.19(a) and Fig. 8.19(b).

In order to evaluate the effect of the imperfect LED driven signal on the extracted fluorescence lifetime, we measured the lifetime of a yellow plastic test slide by using two different LED driven signals: one from MEM-FLIM2 camera output, the other one from the Agilent pulse pattern generator. A green plastic slide with a known lifetime of 2.8 ns was used for the system calibration [135]. The results are shown in Table 8.4. Since there is no clear improvement by using the LED driven signal from the external equipment, we carry out other lifetime experiments using the signal directly from the MEM-FLIM2 camera.

8.5.5 Mask displacement

Experiments have shown that the lifetime derived from the phase change is quite stable, but when the integration time of the experiment is increased, the lifetime derived from the modulation depth change has a tendency to increase. An example is given in the lifetime of GFP labeling fixed U2OS cells. The results are shown in Table. 8.5. A

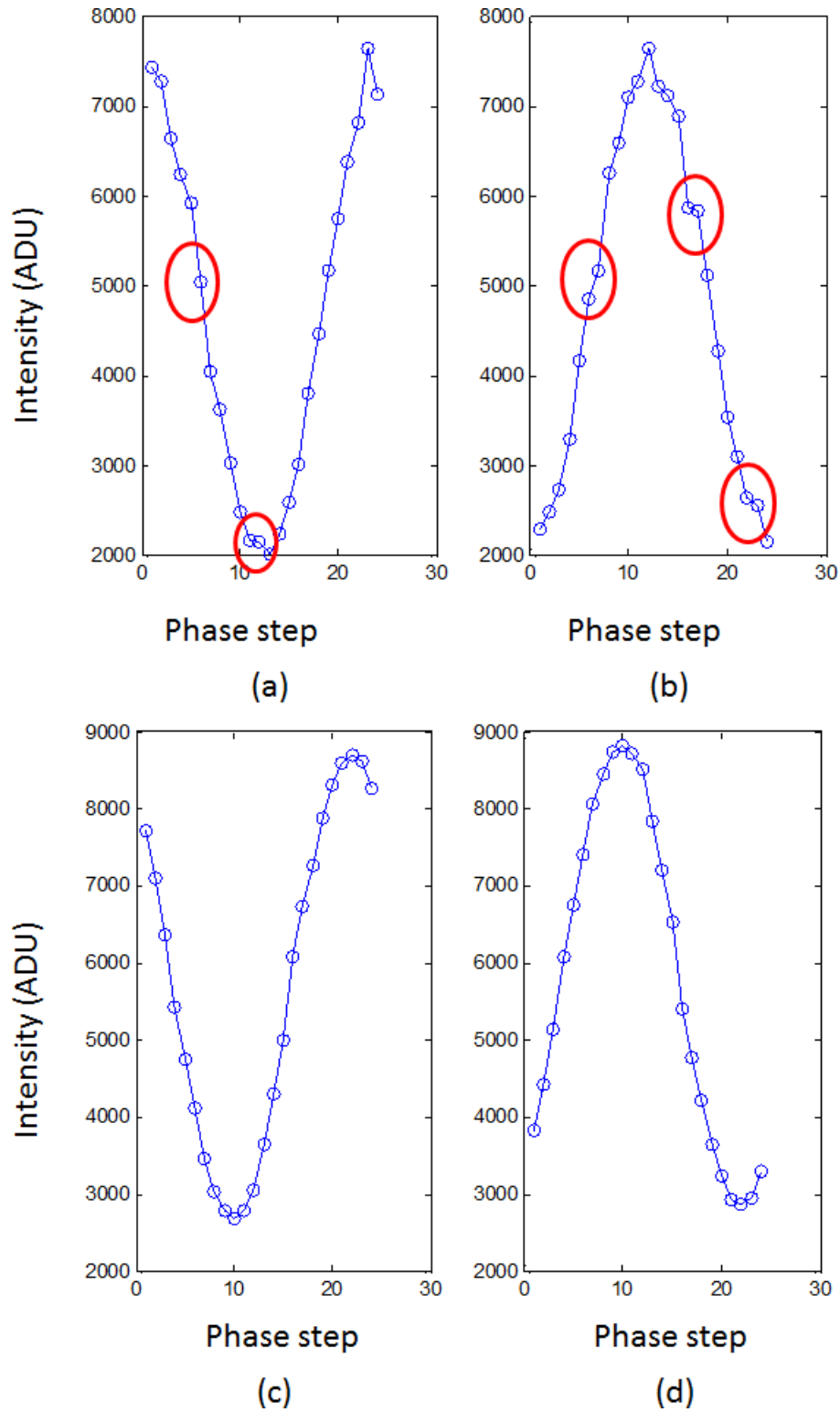


Figure 8.19: The intensity curve at different phase steps. (a) and (b) are two phase images using the MEM-FLIM2 camera output as the LED driven signal, (c) and (d) are two phase images using the Agilent pulse pattern generator output as the LED driven signal.

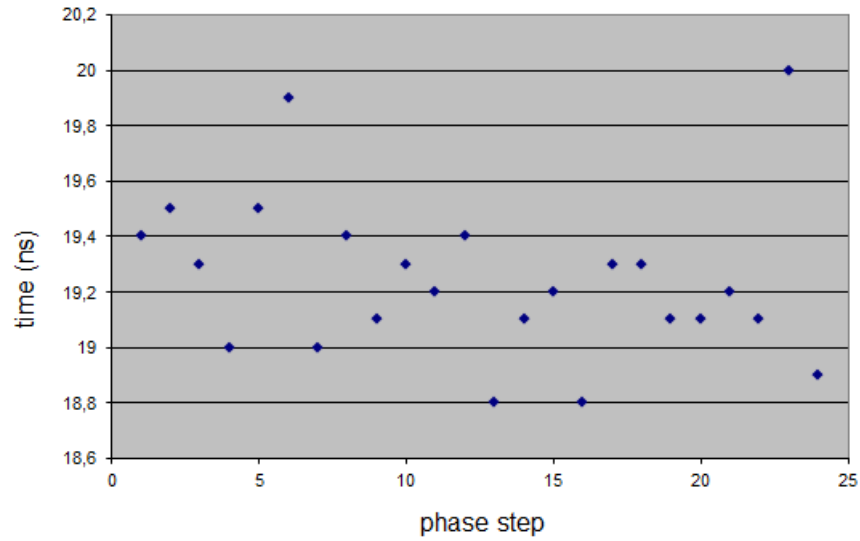


Figure 8.20: The pulse width of the MEM-FLIM2 output LED driven signal at different phase steps.

Table 8.4: The fluorescence lifetime of the yellow plastic slide measured by using two different LED driven signals.

Signal for driven LED	Lifetime _{phase} (ns)	Lifetime _{modulation} (ns)
MEM-FLIM2	5.62 ± 0.40	5.53 ± 0.28
Agilent generator	5.59 ± 0.45	5.51 ± 0.16



(a)



(b)



(c)

Figure 8.21: The waveform of the MEM-FLIM2 output signal which is used to drive LED at (a) normal width, (b) longer width, and (c) shorter width.

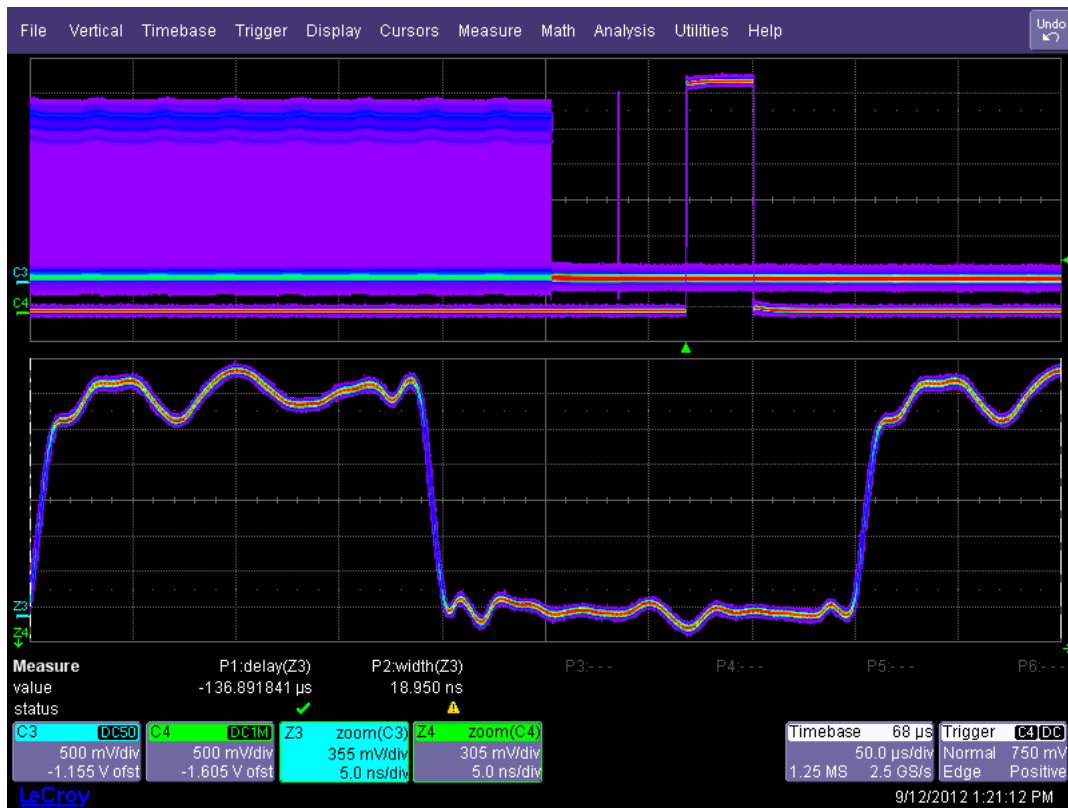


Figure 8.22: Accumulate persistence image of the MEM-FLIM2 output LED driven signal.



Figure 8.23: Waveforms of the toggle gate signal and the LED driven signal.

Table 8.5: The increase in the fluorescence lifetime derived from the modulation depth change with increased integration time.

Integration time (ms)	2000	180	100
Tau-phase (ns)	2.24 ± 0.76	2.41 ± 0.58	2.33 ± 1.29
Tau-modulation (ns)	6.74 ± 1.04	4.29 ± 1.22	2.81 ± 1.57
Modulation	0.38 ± 0.02	0.43 ± 0.03	0.52 ± 0.06

Zeiss oil objective with a magnification of $40\times$ and a numerical aperture of 1.3 was used for this experiment. A 10×10 pixel region was chosen for analyzing the data.

This effect can be explained by a known defect in this version of the MEM-FLIM sensor chip. The MEM-FLIM chip has a mask protecting parts of the surface from exposure to photons. In the current version there is a slight displacement of the mask from its intended position. This means that the photoelectrons that we measure are to a certain extent caused by contributions from the wrong source, resulting in a lower modulation depth. This defect will be corrected in the next version of the sensor chip.

8.6 Discussion and Conclusion

We have designed, built and tested an all-solid-state CCD-based image sensor and camera for fluorescence lifetime (FLIM) imaging. A detailed comparison between the MEM-FLIM and reference cameras is shown in Table 8.6. Using the MEM-FLIM camera, we successfully measured the lifetimes for various fluorescent objects including biological samples.

Table 8.6: Comparison of the MEM-FLIM2 and the reference cameras.

	MEM-FLIM2 camera	Reference camera
Fill factor	44%	>50%
CCD pixel size (μm)	17	20.6*
Active pixel number	212×212	696×520
Modulation frequency (MHz)	25	0.001-120
ADC readout frequency (MHz)	25	11
Sampling density (samples/ μm @ $20\times$)	1.24×1.24	1.07×1.07
OTF @ 500 cycles/mm	0.75	0.39
Sensitivity(ADU/ e^-)	0.43 ± 0.03	0.53 ± 0.03
Detection limit at short integration time(e^-)	51.4	35.4
Bits	14	12
Linearity	0.999995	0.999385
σ_{readout} ADU(e^-)	5.9(13.72)	3.4(5.67)
Dark current (e^-/ms)	0.29	0.08

* The pixel size of the CCD sensor itself is $6.45 \mu\text{m}$, we are using 2×2 binned mode, which gives $12.9 \mu\text{m}$, and the pixels as “projected” onto the photocathode by the fiber optic taper are magnified $1.6\times$, arriving at $20.6 \mu\text{m}$ of effective pixel size of the intensified camera system.

The MEM-FLIM results are comparable to the reference system. There are several advantages for the MEM-FLIM system over the reference system. (1) The camera can be modulated at the pixel level permitting the recording of two phase images at once. The acquisition time can thus be shortened by using the MEM-FLIM camera, which causes less photobleaching in the biological sample. (2) The MEM-FLIM camera does not need high voltage sources and RF amplifiers and the system is more compact than the reference system. (3) In the MEM-FLIM system, one can change the integration time and the analog gain which has no effect on the optical system itself. In the conventional

frequency domain FLIM system, one needs to control both the integration time and the MCP voltage in order to make use of the full dynamic range of the camera. However, changing the MCP voltage by more than approximately 50 V (depending on the intensifier and the MCP voltages used) means changing the system itself, which in turn means that the calibration done at another MCP voltage is no longer reliable. So one needs to pay extra attention when adjusting the settings on the conventional frequency domain FLIM system. (4) Possible sources of noise and geometric distortion are significantly reduced. (5) The image quality from the MEM-FLIM camera is much better than the conventional intensifier-based CCD camera and the MEM-FLIM camera thereby reveals more detailed structures in the biological samples. (6) The quantum efficiency of the MEM-FLIM camera is much higher than the reference camera. For the MEM-FLIM camera, the quantum efficiency is determined by the characteristics of the front illuminated CCD, about 30%, 50% and 70% at 500nm, 600nm and 700nm, respectively. For the reference camera, the quantum efficiency of the photo cathode at 500 nm is around 11%. Further, there are losses in other parts of the system including the fiber optics and the CCD camera, not all of which can be attributed to true quantum effects.

It is also interesting to compare our results to the previously developed CCD camera described in [123, 128], as shown in Table. 8.7. Both the SR-2 and the MEM-FLIM cameras are able to measure fluorescence lifetimes, and the modulation depth and the lifetime results are comparable. The quantum efficiencies of the two cameras are comparable since they are both determined by the characteristics of a front illuminated CCD. There are big improvements in the MEM-FLIM camera compared with the SR-2 camera. Although both the MEM-FLIM and the SR-2 cameras are non-cooled camera, we can see clear influence of the dark current on the SR-2 camera. The presence of an edge artifact in the phase images in Fig. 2 (e,f) of [123] and Fig. 3 of [128] can be attributed to the dark current. In the MEM-FLIM camera, however, there is a uniform phase response across the sensor and the dark current influence can be ignored. The MEM-FLIM camera has more than twice as many pixels, smaller pixel sizes for better spatial sampling density, and a fill factor that is 2.75 times that of the SR-2. The modulation frequency of the MEM-FLIM camera described in this manuscript is 25 MHz, while the SR-2 camera is 20 MHz. As mentioned in [123, 128], the modulation frequency can, in principle, be significantly increased for both cameras but all measurements of camera performance would have to be re-evaluated for any higher frequency. At this time we can only compare performance at the frequencies that have been used.

8.7 Future work

The MEM-FLIM cameras are able to measure the fluorescence lifetime, but the modulation frequency is now limited to 25 MHz. We intend to achieve higher modulation frequencies in the next generation camera. The next generation camera will also have larger pixels (better light gathering) and more pixels (larger field-of-view) compared to the current design. Improved chip-level mask design should improve the modulation

Table 8.7: Comparison of the MEM-FLIM camera and the SR-2 camera.

	MEM-FLIM2	SR-2
Sensor type	CCD	CCD/CMOS hybrid
Pixel number	212×212	124×160
Pixel size (μm)	17×17	40×55
Fill factor	44%	16%
Modulation frequency (MHz)	25	20
Measured GFP lifetime (phase)	2.6 ± 0.4	2.6 ± 0.4
Measured modulation depth	$55 \pm 2\%$	$50 \pm 3\%$
Dark current influence	can be ignored	cannot be ignored

depth.

The camera is not perfect and there is still room for improvement.

8.8 Acknowledgments

Funding from Innovation-Oriented Research Program (IOP) of The Netherlands (IPD083412A) is gratefully acknowledged. We thank Dr. Vered Raz of the Leiden University Medical Center for providing us with the U2OS cells.

MEM-FLIM architecture revisited

Abstract

Since the MEM-FLIM1 camera suffers from a low charge transfer efficiency, the architecture used by the MEM-FLIM2 (toggling in the vertical direction) was chosen to carry out the fluorescence lifetime experiments in the previous chapter. Based on the evaluation of the two prototypes, the vertical toggle concept has been chosen for the next prototype, the MEM-FLIM3 camera. Several improvements have been made in the sensor design for the MEM-FLIM3 camera, such as higher fill factor, greater number of pixels etc. The MEM-FLIM3 camera is able to operate at higher frequencies (40, 60 and 80 MHz) and has an option for electron multiplication. In this chapter, details of the architecture improvements are presented and discussed.

Keywords: Vertical toggling, electron multiplying CCD, higher frequency

9.1 Introduction

Two prototypes of the MEM-FLIM cameras have been evaluated, and the architecture design from the MEM-FLIM2 camera (vertical toggling) has been chosen for the third generation prototype. Due to the fact that the light shield over the vertical charge storage areas was designed too narrow in the MEM-FLIM1 camera, the charge separation was not optimal. Furthermore, the vertical transport efficiency of the MEM-FLIM1 sensor was not up to standard, which made it impossible to properly image biological samples. Compared with the MEM-FLIM1 camera (horizontal toggling), the MEM-FLIM2 camera has a bigger fill factor and simpler design. When using the MEM-FLIM2 camera, the incident light must be eliminated during the readout due to its full-frame CCD design. This disadvantage is avoided by using a properly designed LED, which is switched off during readout.

The results on the biological samples have shown that the MEM-FLIM2 camera is qualified for measuring fluorescence lifetime. There is, however, still quite some room for improvement. The limitations of using the MEM-FLIM2 camera to measure sample fluorescence lifetime are presented in the following section.

9.2 Limitations of MEM-FLIM2

9.2.1 Frequency

One of the biggest limitations of the MEM-FLIM2 camera is the modulation frequency, which is fixed at 25 MHz. First of all, when the frequency of the camera is limited to one value, it cannot be used to measure different lifetime components in a multi-component fluorescence lifetime system. Multiple frequencies are needed in order to do so, as described in Chapter 3. Second, this locked frequency (25 MHz) is not always the optimal frequency for different biological samples with various lifetimes.

In order to determine the optimal modulation frequency, we need to first look at the errors in an estimated lifetime which result from an error in the estimated phase or the modulation depth. The lifetime derived from the phase and the modulation depth are based upon Eq. (9.1) and (9.2):

$$\tau_\theta = \frac{1}{\omega} \tan(\theta) \quad (9.1)$$

$$\tau_m = \frac{1}{\omega} \sqrt{\frac{1}{m^2} - 1} \quad (9.2)$$

If there is an error in the phase estimate $\delta\theta$ or an error in the modulation depth estimate δm , then the errors of the lifetime at an error-free frequency are given by Eq. (9.3) and (9.4):

$$\delta\tau_\theta = \delta\theta \cdot \frac{\partial\tau_\theta}{\partial\theta} = \delta\theta \cdot \frac{1 + \omega^2 \cdot \tau^2}{\omega} \quad (9.3)$$

$$\delta\tau_m = \delta m \cdot \frac{\partial\tau_m}{\partial m} = -\delta m \cdot \frac{(1 + \omega^2 \cdot \tau^2)^{3/2}}{\omega^2 \cdot \tau_m} \quad (9.4)$$

Given a lifetime and an error in the phase or modulation depth, the optimal frequencies can be derived when $d\delta\tau_\theta/d\omega = 0$ or $d\delta\tau_m/d\omega = 0$, which result in Eq. (9.5) and (9.6):

$$\omega_\theta = \frac{1}{\tau} \quad (9.5)$$

$$\omega_m = \frac{\sqrt{2}}{\tau} \quad (9.6)$$

Using Eq. (9.5) and (9.6), we can calculate that a frequency of 25 MHz is suitable for measuring samples with a lifetime of $1/(2 \cdot \pi \cdot 25(\text{MHz})) \approx 6.4$ ns (for the lifetime derived from the phase change) or $\sqrt{2}/(2 \cdot \pi \cdot 25(\text{MHz})) \approx 9$ ns (for the lifetime derived from the modulation depth change). Assuming the biological sample with a lifetime of 2.5 ns (the typical fluorescence lifetime of GFP is 2-3 ns [122, 141]), the optimal modulation frequencies then will be $1/(2 \cdot \pi \cdot 2.5(\text{ns})) \approx 64$ MHz (for phase) or $\sqrt{2}/(2 \cdot \pi \cdot 2.5(\text{ns})) \approx 90$ MHz (for modulation depth), which are far away from 25 MHz.

So for the next MEM-FLIM prototype, we would like the camera be able to modulate at higher frequencies and more frequencies.

9.2.2 Power consumption

Another major concern for the MEM-FLIM1 and MEM-FLIM2 cameras is the power consumption of the imager and the required hardware to drive the on-chip capacitances. The limitation for the low modulation frequency in the MEM-FLIM2 camera is the power consumption of the chip. As shown in Chapter 8, when the sensor is modulated at 20 MHz, the temperature at the driver can go up to 92 degrees. The high temperature on the chip not only will affect the dark current of the sensor, but also shorten the device lifetime. The architecture needs to be improved in order to be able to modulate at higher frequencies without worrying about the thermal damage to the chip.

9.2.3 Field of view

As shown in the intensity and lifetime images from the MEM-FLIM2 camera versus the reference camera in Chapter 8, one can notice that the field of view from the MEM-FLIM2 camera is significantly smaller than that of the reference camera. For example, when an objective with a magnification of $20\times$ and a numerical aperture of 0.5 was used, a 10×10 pixel area corresponded to an area of $(1/1.07 * 10)^2 = 87 \mu m^2$ for the reference camera and $(1/1.24 * 10)^2 = 65 \mu m^2$ for the MEM-FLIM2 camera. 1.07 and 24 are the sampling density of the two cameras in “samples/ μm ”. The number of pixel in the reference camera is approximately 8 times that of the MEM-FLIM2 camera. The area covered by the reference camera (3.1 mm^2) is 10 times the area covered by the MEM-FLIM2 camera (0.3 mm^2). A larger field of view makes the observation area bigger, and the collection of data more efficiently.

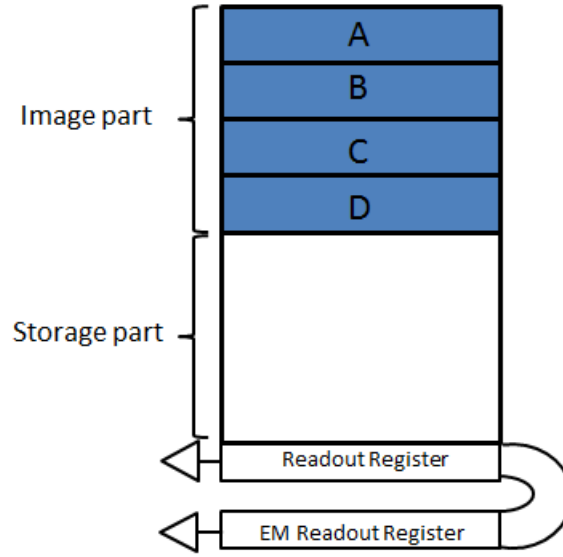


Figure 9.1: The MEM-FLIM3 sensor design.

9.2.4 Low light performance

In the MEM-FLIM systems, demodulation is carried out at the pixel level instead of at an image intensifier as in the conventional FD-FLIM. The other function of the image intensifier - amplifying the detected light - cannot, however, be neglected. In order to make the MEM-FLIM camera a successful commercial product in the future, a better low light performance is required.

9.3 MEM-FLIM3 design

By comparing the performances of the MEM-FLIM1 and MEM-FLIM2 systems, the vertical toggling technique used in the MEM-FLIM2 sensor was chosen for the next generation sensor: MEM-FLIM3. The MEM-FLIM3 sensor is a frame transfer CCD sensor with 512×512 active pixels each of $24 \times 24 \mu\text{m}$, with a storage area $1024(\text{V}) \times 512(\text{H})$ cells of $12(\text{V}) \times 24(\text{H}) \mu\text{m}$. The sensor design is shown in Fig. 9.1. In order to lower the on-chip power consumption at high toggling frequencies, the image section has been split into four vertical sections with separate gate connections for the high-frequency gates. All high-frequency interconnects, on the chip and in the package, were made as identical as possible to achieve identical performance for all four image sections when demodulating. The split enables the MEM-FLIM3 to operate at high modulation frequencies. Compared to 25 MHz for the MEM-FLIM2 camera, MEM-FLIM3 can be modulated at 20, 40, 60, and 80 MHz. The images of the MEM-FLIM3 camera and the assembled sensor are shown in Fig. 9.2.

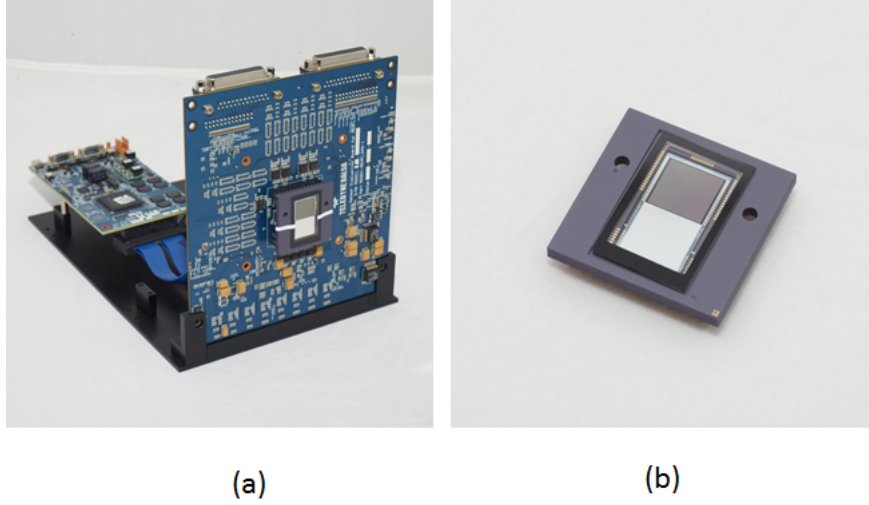


Figure 9.2: (a) The MEM-FLIM3 camera and (b) the assembled sensor.

9.3.1 Pixel design

Not only the pixel size and number are different from the pixels on the image part and the storage part of the MEM-FLIM3 sensor, design concepts are also different. The pixels in the image part are toggled with high frequency signals while the pixels in the storage are straightforward 4-phase pixels for transport and storage only.

9.3.1.1 Photogate design

To collect as many incident photons as possible we must maximize the fill factor. The large pixel size ($24\ \mu\text{m}$), however, will result in a low electric field even at high voltage swings of the neighboring toggle gates. The generated photo electrons might not be able to travel to the storage gates in a low electric field at higher toggling frequencies. This challenge was solved by splitting each photo-gate into three parts: a central part which is not clocked, and two ‘side-wings’ clocked at a reduced voltage swing, as shown in Fig. 9.3. Each pixel will have four toggle gates: two normal toggle gates (TG1 and TG2) like the design in MEM-FLIM2 camera, and two toggling photo gate “wings” (PG1 and PG2). This is done in order to use lower voltages to create the electrical field that will drive the generated electrons to the storage gates (SG1 and SG2). This design was proposed and produced by our project partner Teledyne DALSA.

9.3.1.2 Storage part

In the MEM-FLIM3 sensor, the lower part is the shielded storage part. The storage pixel is a straight forward 4-phase pixel with a size of $24\ \mu\text{m}$ in the horizontal direction and $12\ \mu\text{m}$ in the vertical direction. In order to transport charge from one pixel to

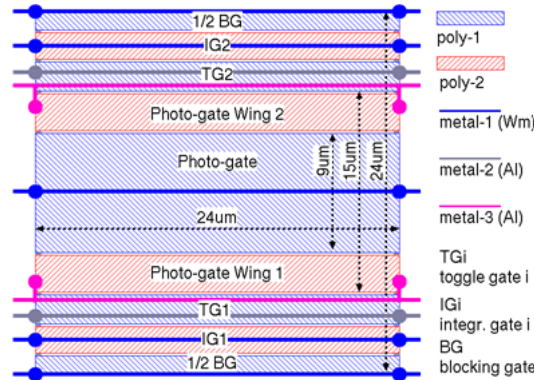


Figure 9.3: The pixel level design of the MEM-FLIM3 sensor.

the next, various charge transport systems are used in applications, such as classic 4-phase system[144], 3-phase system[145], 2-phase system[146], 1-phase system[147], etc. The basic principle is to change the voltages on the gates in order to generate different potentials below the gates. A potential well will be introduced by applying a higher voltage on the gate while a potential barrier will be formed when the applied voltage is lower. The electrons will then “flow” in the potential wells according to the proper design of the clock signal applied to the gates. A N-phase system requires N polysilicon gate electrodes in each pixel cell and takes N steps to finish charge transport from one pixel to the next. In the MEM-FLIM2 camera, a 3-phase transport pulse pattern was used in order to transport the charge to the horizontal register while the MEM-FLIM3 camera uses a 4-phase transport scheme. Figure 9.4* shows the different charge transport schemes of a 3-phase system (a) and a 4-phase system (b).

9.3.2 Horizontal register design

9.3.2.1 EM principle

An electron multiplying (EM) register is a gain register that can generate many thousands of output electrons from a small number of input electrons by impact ionization in a way similar to an avalanche diode. Impact ionization is a process where one energetic charge carrier (in this case an electron) loses energy by creating other charge carriers. The register has several hundred stages, through which the charges generate secondary electrons. The principle of the EM register is shown in Fig. 9.5†. The difference between a standard shift register and an EM register is that the full-well capacity in an EM register is increased and higher clock voltages are applied at selected transfer electrodes to accelerate electrons. A sufficiently high potential difference enables the impact ionization process. The EM register multiplies the signal before the readout noise from the amplifier

*Image source: <http://learn.hamamatsu.com/articles/fourphase.html>. 30 May, 2013.

†Image source: http://www.emccd.com/what_is_emccd/. 10 June, 2013.

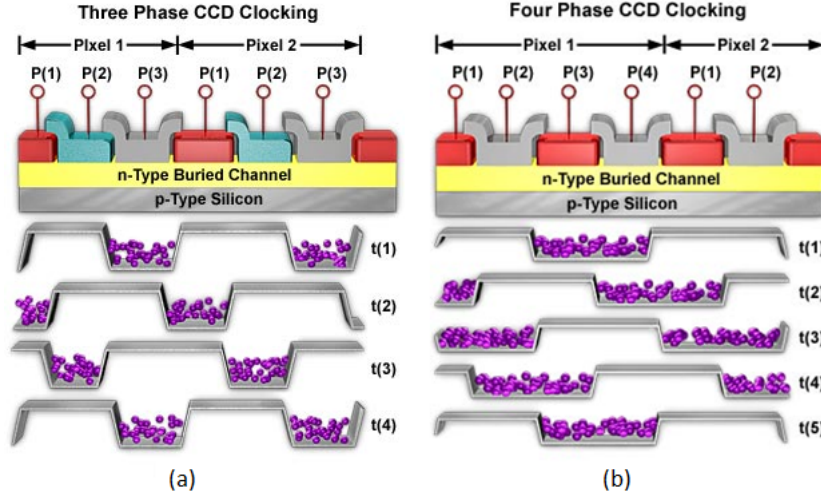


Figure 9.4: The principle of charge transport systems: (a) a 3-phase system and (b) a 4-phase system.

is added, thus the advantage of using an EM register is to improve the signal-to-noise ratio when the signal is below the readout noise floor. The total gain (G_{em}) of an EM register is given by Eq. (9.7) where p_e is the secondary electron generating probability and N_{em} is the stage number in the EM register. p_e depends on the EM clock voltage levels and the CCD temperature. It typically ranges from 0.01 to 0.016[148]. If the secondary electron generating probability is 0.01, with $N_{em} = 1072$, the produced EM gain $G_{em} = 1.01^{1072} = 42905$.

$$G_{em} = (1 + p_e)^{N_{em}} \quad (9.7)$$

9.3.2.2 MEM-FLIM3 EM design

The MEM-FLIM3 camera has two registers: a standard register just below the storage section, like the one in the MEM-FLIM2 camera, and an EM register with 1072 stages below the standard register, as shown in Fig. 9.1. Readout is either through a conventional CCD readout register or through an EM-CCD register. In order to readout charge through the EM registers below, the standard register is designed to be bi-directional. From the standard register, the charges can be readout directly from the left side, or transferred in to the EM register from the right side. The standard register has 556 register cells with a pitch size of $24 \mu\text{m}$ in order to match the storage pixel size. The EM register has 1112 EM cells with a pitch size of $12 \mu\text{m}$. The differences between the standard register and EM register used in MEM-FLIM3 camera are listed in Table 9.1. A scanning electron microscope (SEM) image of the details of EM-CCD register are shown in Fig. 9.6.

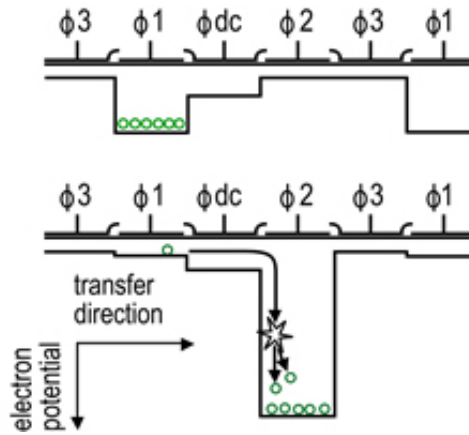


Figure 9.5: The principle of the EM register.

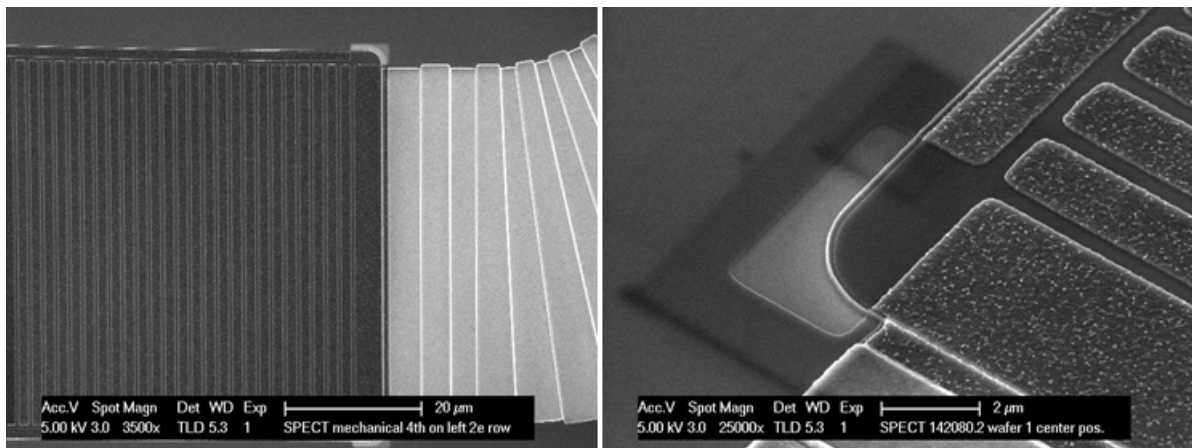


Figure 9.6: The SEM photos of details of EM-CCD register.

Table 9.1: The difference between the standard register and EM register used in MEM-FLIM3 camera.

	Standard register	EM register
Number	556	1112
Pitch size [μm]	24	12
Direction	bi-directional	uni-directional
Charge transport system	3-phase	6-phase

9.4 Conclusion

A third-generation version of a direct pixel-modulated CCD camera- MEM-FLIM3- has been developed for FLIM application. The comparisons between the MEM-FLIM2 and the MEM-FLIM3 cameras are shown in the Table 9.2. Compared to the MEM-FLIM2 camera, several parameters of the MEM-FLIM3 camera have been improved, such as the pixel number, modulation frequency, fill factor, and full well capacity. Like the MEM-FLIM2 sensor, the MEM-FLIM3 sensor is vertically toggled. The toggling mechanism in the MEM-FLIM3 camera, however, is more complicated than the one in the MEM-FLIM2 camera. Due to the larger pixel size in the MEM-FLIM3 camera, extra toggling are added on the pixels in order to help the generated photo electrons to travel to the desired storage gate in time. The image section in the MEM-FLIM3 camera is divided into four horizontal parts to allow more drivers to share the load. The capacitor per pin can be minimized in this way. The influences of these modifications will be addressed in the next chapter.

Table 9.2: Design comparison of the MEM-FLIM2 and the MEM-FLIM3 cameras.

	MEM-FLIM2	MEM-FLIM3
CCD architecture	full-frame CCD	frame transfer CCD
Imaging CCD pixel size (μm)	17	24
Active Imaging pixel number	212×212	512×512
Storage pixel	No	$512(\text{H}) \times 1024(\text{V})$
Storage pixel size (μm)	—	$24(\text{H}) \times 12(\text{V})$
Fill factor	44%	50%
Modulation frequency (MHz)	25	20,40,60,80
ADC readout frequency (MHz)	25	20
Full well capacity (ke^-)	38	67
Bits	14	14
Charge transport pattern	3-phase	4-phase
EM function	No	Yes

Evaluation of the new MEM-FLIM3 architecture

Abstract

The performance of the MEM-FLIM3 camera at different modulation frequencies is evaluated using the methods described in chapter 7. The comparisons between the MEM-FLIM3 camera with the previous two versions of MEM-FLIM cameras together with the reference camera are presented. The fluorescence lifetime measurements using the MEM-FLIM3 system are also presented and discussed in this chapter.

Keywords: FLIM, all-solid-state camera, pixel modulation, camera evaluation and comparison

10.1 Introduction

The same methods used to evaluate the MEM-FLIM1 and MEM-FLIM2 cameras are applied to the MEM-FLIM3 camera. Unlike the previous single frequency modulated MEM-FLIM cameras, the MEM-FLIM3 camera can be modulated at four different frequencies (20, 40, 60, 80 MHz). At each frequency, the MEM-FLIM3 camera has a distinct configuration file in order to optimize the performance. Quantitative measurements are performed at four different modulation frequencies.

10.2 System configuration and materials

The system configuration remains the same as described in Chapter 8, except for: (1) LED (LXML-PR01-0500, LUXEON, REBEL), which has the peak wavelength at 460 nm, is used for the MEM-FLIM3 camera. The LED can be controlled (modulated) both by the reference FLIM system and the MEM-FLIM3 system at frequencies upto 80 MHz, and (2) necessary cooling elements are added to the MEM-FLIM3 camera to improve the dark current performance. In addition to (a) a small mechanical fan is used to assist air circulation inside of the camera housing similar to the setup for the MEM-FLIM2 camera, (b) an aluminum plate is mounted beneath the sensor board with the aim of conducting the sensor heat, (c) if necessary, two Peltier cooling units (Farnell 1639748, MCPE-071-10-13, 19.1 W) and two heat sinks (Farnell 1669148, ATS-58002-C1-R0) can be attached to the metal plate. The schematic diagram of the added cooling elements is shown in the Fig. 10.1(a) and the experimental setup is shown in Fig. 10.1(b). The temperature of the sensor with the Peltier cooling unit can be held at 18°C.

An oscilloscope (LeCroy WAVESURFER 64Xs, 300MHz) is used to monitor the waveforms from the MEM-FLIM3 camera. The LED by default is driven directly by the MEM-FLIM3 camera. It can also be driven, however, by an Agilent (81110A) pulse pattern generator to obtain a more stable signal for comparison.

10.3 Camera characteristic - Performance

The methods for evaluating the MEM-FLIM3 camera are identical to the ones used for the MEM-FLIM2 camera, which are explained in Chapters 7 and 8. Similar measurement figures will not be shown here for the MEM-FLIM3 camera but the data will be presented and discussed. For the camera evaluations, unless specified otherwise, the camera is operated at 24°C without Peltier cooling. This will be explained in the section on dark current, in section 10.3.3.2).

10.3.1 Linearity

Since the image has been split into four different vertical sections as shown in Fig. 9.1, we chose to examine identically sized regions from each section. Since every pixel has

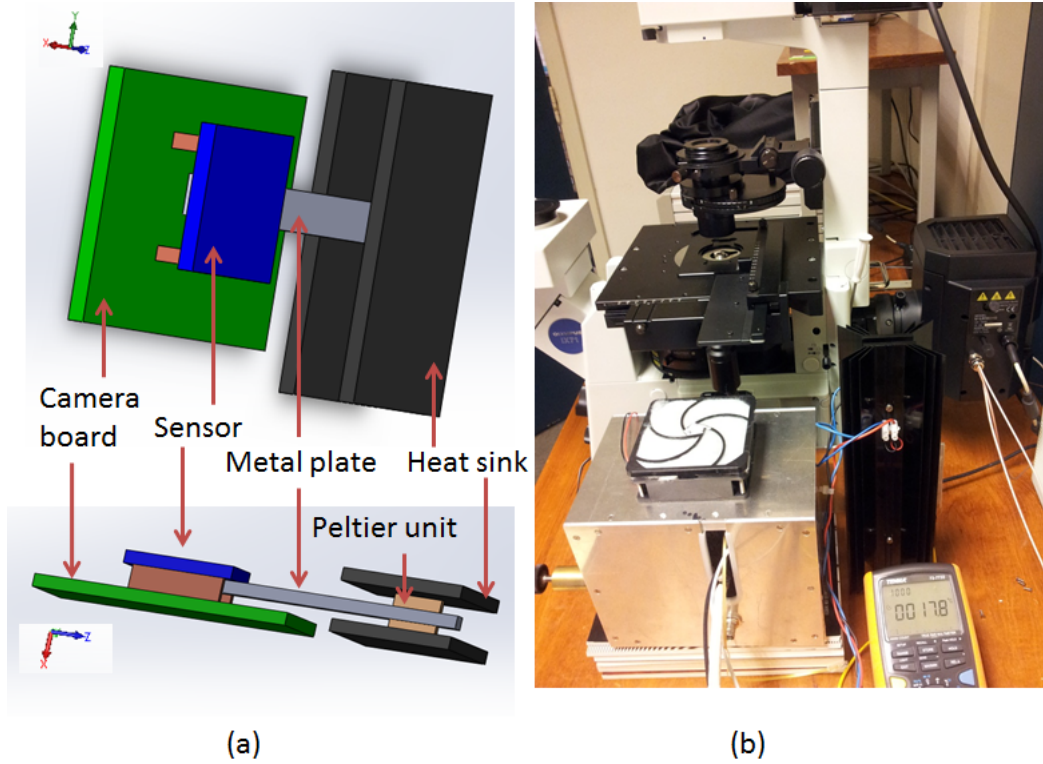


Figure 10.1: The cooling elements added to the MEM-FLIM3 camera. (a) the schematic diagram of the added cooling elements, and (b) the experimental setup.

two phase registers, each of which contributes to one phase image. As a result, the whole image consists of two phase images: “phase one” and “phase two”. The MEM-FLIM3 shows linear photometric response at all four frequencies. All four parts of the image show good linearity. The photometric response is linear up to almost full dynamic range. The average value of the regression coefficient of the intensity versus integration time curve of the MEM-FLIM3 is 0.999905 ± 0.000132 .

10.3.2 Resolution

The horizontal and vertical OTF performances are quite comparable at all four frequencies, an example at 20 MHz is shown in Fig. 10.2. The OTF comparison of the MEM-FLIM3 at different frequencies is shown in Fig. 10.3. The OTF performance of the MEM-FLIM3 camera is quite consistent regardless of the frequency. The OTF in Fig. 10.3 for each frequency is the average value of the OTF at horizontal and vertical directions. One might expect that mounting a mechanical fan on the camera housing may degrade the image quality. Figure 10.4 shows, however, that the influences of the fan can be neglected. The comparison between the MEM-FLIM3 camera with the MEM-FLIM2 and reference camera is shown in Fig. 10.5. Even though the MEM-FLIM3

Table 10.1: Linearity performance of the MEM-FLIM3 camera.

Region	Phase	20 MHz	40 MHz	60 MHz	80 MHz
A	one	0.999955	0.999957	0.999987	0.999993
[50:100,260:310]	two	0.999996	0.999998	0.999995	0.999995
B	one	0.999975	0.999976	0.999987	0.999999
[150:200,260:310]	two	0.999991	0.999986	0.999982	0.999985
C	one	0.999853	0.999823	0.999943	0.999998
[300:350,260:310]	two	0.999573	0.999694	0.999894	0.999946
D	one	0.99985	0.999804	0.999854	0.999948
[410:460,260:310]	two	0.999454	0.999697	0.999929v	0.999954
Average		0.999831	0.999867	0.999946	0.999974

camera shows a lower OTF compared to the MEM-FLIM2 camera due to a bigger pixel size, it still outperforms the intensifier-based reference camera, the result of which will be further confirmed by the quality of the biological sample image obtained both from the MEM-FLIM3 and the reference camera in section 10.4 .

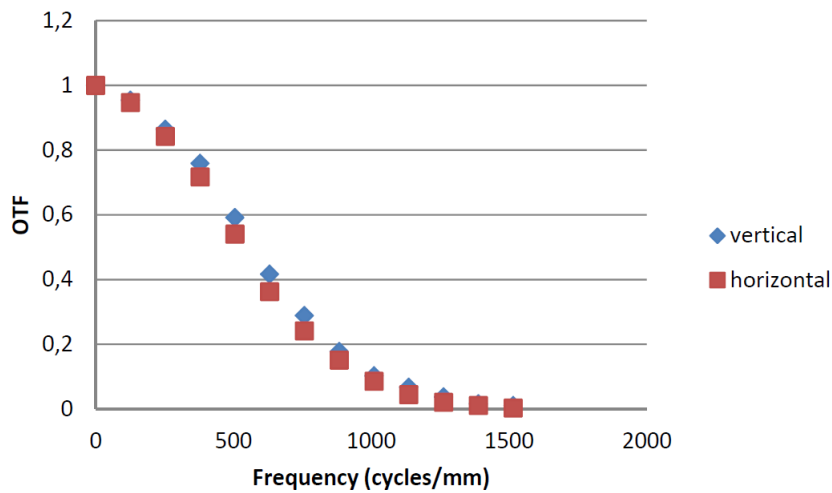


Figure 10.2: The OTF comparisons between vertical and horizontal directions of the MEM-FLIM3 camera modulated at 20 MHz.

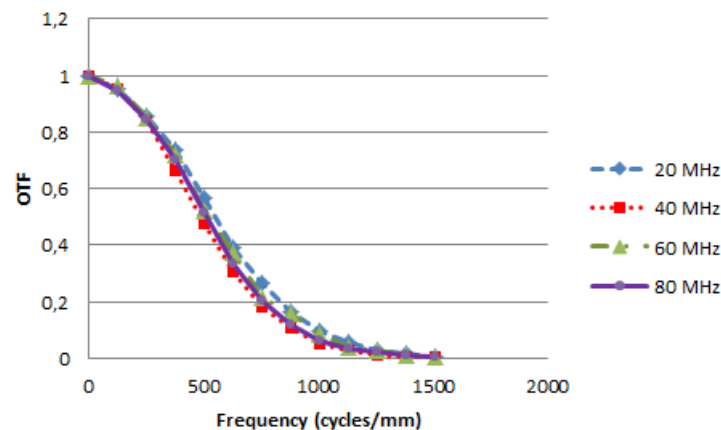


Figure 10.3: The OTF comparisons between different modulation frequencies of the MEM-FLIM3 camera.

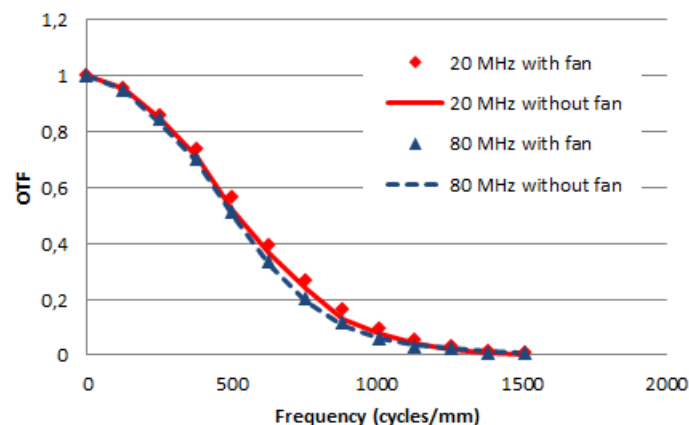


Figure 10.4: The OTF comparisons with and without the mechanical fan.

10.3.3 Noise

10.3.3.1 Poisson distribution

The Poisson distribution model of the noise source has been validated for all four frequencies. An example is shown in Fig. 10.6 when the modulation frequency for the MEM-FLIM3 camera was set to 80 MHz. The integration time was 40 ms. The linear fit shows that the noise source in the image is Poisson distributed.

Checking the Poisson distribution is crucial for evaluating a camera. During our evaluations, there were situations when the noise distribution was not entirely Poisson distributed, as shown in Fig. 10.7. At higher intensity values, the variance of the difference image was no longer linear with the mean intensity. The modulation frequency in this

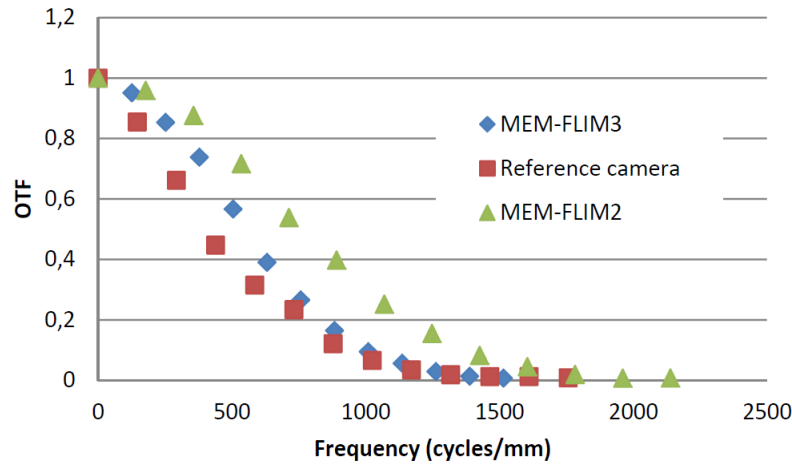


Figure 10.5: The OTF performance of the MEM-FLIM3 camera compared with the MEM-FLIM2, and the reference camera.

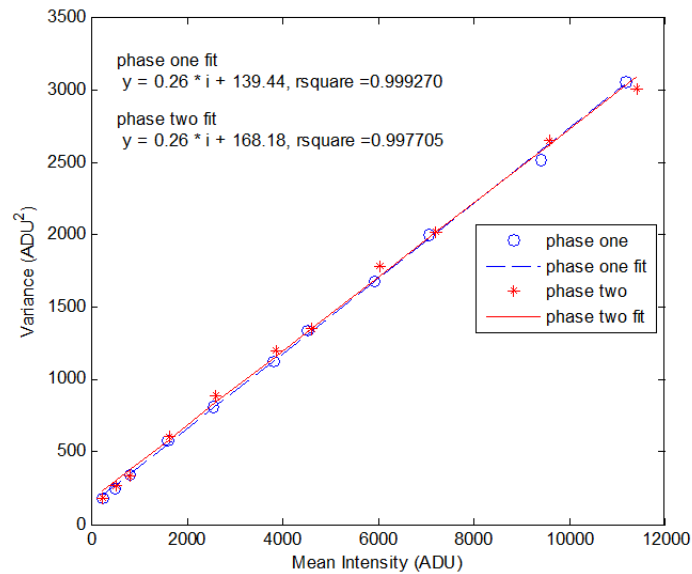


Figure 10.6: The Poisson assumption validation and the sensitivity of the MEM-FLIM3 camera.

figure was set to 20 MHz and we noticed this phenomenon on all four frequencies. This was caused by incorrect camera hardware (in our case a wrong resistor) or imperfect voltage configurations on the gates which effect charge transport for larger charge packages. This resulted in a limitation of the usable dynamic range. We could only use one third of the full dynamic range (from original 16383 ADU to around 5000 ADU). Lifetime measurements

at higher intensity range were hampered. After identifying the reason for non-Poisson distribution at higher intensity, an improved performance of the camera was achieved, as shown in Fig. 10.6 above.

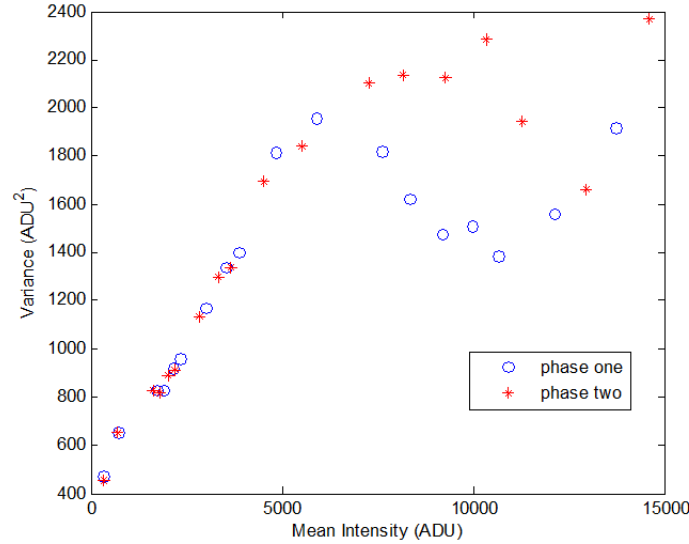


Figure 10.7: The noise distributions of the MEM-FLIM3 camera at imperfect settings.

10.3.3.2 Dark current noise

Linearity in integration time

The dark current generated from the MEM-FLIM3 camera is linear in the integration time, as shown in Fig. 10.8. In this figure, the camera is operated at 40 MHz. When the integration time is 600 ms, the dark current is $285/16383 \approx 1.7\%$ of the full dynamic range. Since the electron to ADU converting factor is known from the absolute sensitivity experiment, which is 0.256 ADU/e^- , the dark current can also be written as $285 \text{ (ADU)} / 0.256 \text{ (ADU/e}^-) / 600 \text{ (ms)} = 1.86 \text{ e}^-/\text{ms}$.

The slopes of the fitting for the dark image intensity (ADU) versus integration time (ms) at different frequencies are shown in the Table 10.2. The dark current values of the MEM-FLIM3 camera are 1.75, 1.86, 1.93, and 1.94 (e^-/ms) at 20, 40, 60 and 80 MHz, respectively.

Peltier cooling

The dark current noise, however, is dependent on which MEM-FLIM3 camera is being evaluated. There are cameras which yield worse performance. For one MEM-FLIM3 camera which we evaluated, the temperature of the sensor can go up to 50°C

Table 10.2: The slopes of fitting for the dark image intensity (ADU) versus integration time (ms) at different frequencies.

Phase	20 MHz	40 MHz	60 MHz	80 MHz
One	0.11791	0.14816	0.15158	0.17067
Two	0.10823	0.14747	0.15936	0.18780
Average	0.113	0.148	0.155	0.179

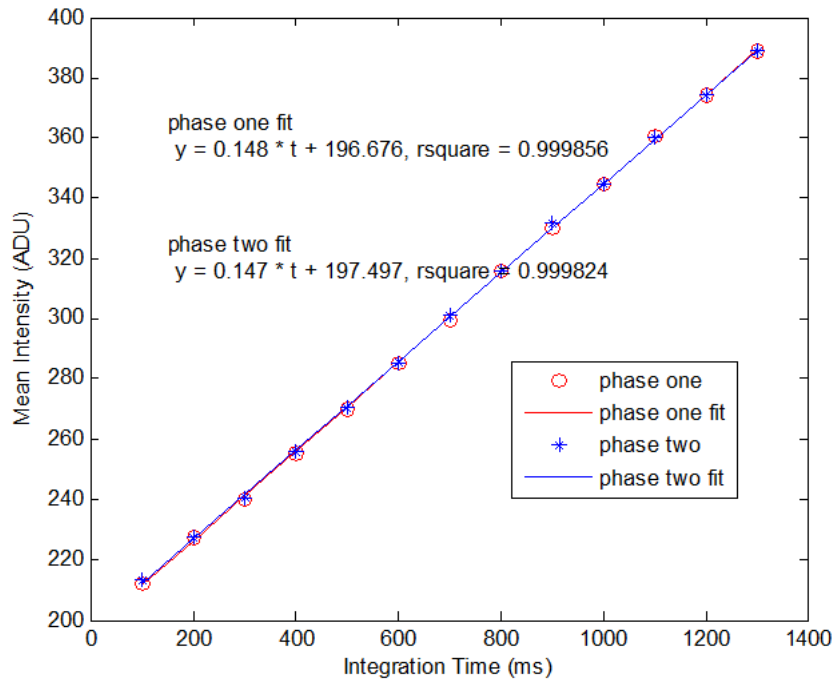


Figure 10.8: The linear relation between dark current with integration time.

at higher frequencies after the camera has been switched on for 10 minutes, as shown in Fig. 10.9. The differences of the final temperature which the sensor will reach at different frequencies are the results of a combination of factors such as the toggling frequency, the amplitude and shape of the toggle gate and toggle photogate signal. The generated dark current cannot be neglected as it limits the useful range of the camera. For example, at 500 ms integration time at 80 MHz, the dark current can go up to $7.38(e^-/\text{ms}) * 500(\text{ms}) / 0.255(\text{ADU}/e^-) = 941(\text{ADU}) \approx 1000(\text{ADU})$. The value of $0.255(\text{ADU}/e^-)$ is the sensitivity measured for this camera, as explained in section 10.3.4.1. In this case, one extra measure was taken when using the MEM-FLIM3 camera,

the attachment of the Peltier cooling units to the aluminum metal plate. The sensor temperature in this setup stabilized at 18 °C throughout the experiments with the Peltier cooling units. The dark current for the cooled MEM-FLIM3 camera improved when compared to the non-cooled MEM-FLIM3 camera, as shown in Table 10.3. For example, at 500 ms integration time at 80 MHz, the dark current decreases from 1000 (ADU) to $2.57(e^-/\text{ms}) * 500(\text{ms})/0.255(\text{ADU}/e^-) = 327(\text{ADU}) \approx 300(\text{ADU})$.

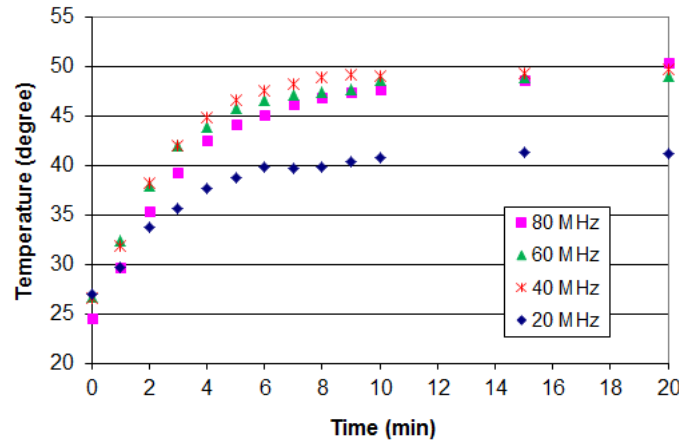


Figure 10.9: The sensor temperature without cooling unit.

Table 10.3: The dark current of one MEM-FLIM3 camera before and after Peltier cooling.

Cooling	20 MHz	40 MHz	60 MHz	80 MHz	Mean
Before (e-/ms)	3.28	5.43	8.29	7.38	6.10
After (e-/ms)	1.83	2.37	3.03	2.57	2.45

Dark current pattern

The dark current for all the MEM-FLIM cameras, however, has a similar pattern as shown in Fig. 10.10. This is the dark current image when the camera is operated at 1000 ms integration time at 20 MHz. The middle of the dark image displays a higher dark current. The dark current is calculated from the middle dark current region for camera comparison. The higher dark current is most probably related to the additional processing step required for the MEM-FLIM3 camera to integrate the aluminum layer with the anti-reflection (AR) layer. The AR layer in the MEM-FLIM3 camera is a layer to shield the dark pixels on the edge of the image from light. In the MEM-FLIM1 and MEM-FLIM2

cameras the dark pixels on the edges of the image are shielded with an aluminum layer, so no AR layer is needed. For the MEM-FLIM3 camera, the aluminum layer is, however, used to connect the toggle gates and toggle photogates. Thus the AR layer is a necessity for the MEM-FLIM3 camera. In order to pinpoint the actual source for the higher dark current in the middle of the image, extra experiments need to be carried out in the wafer fabrication facility.

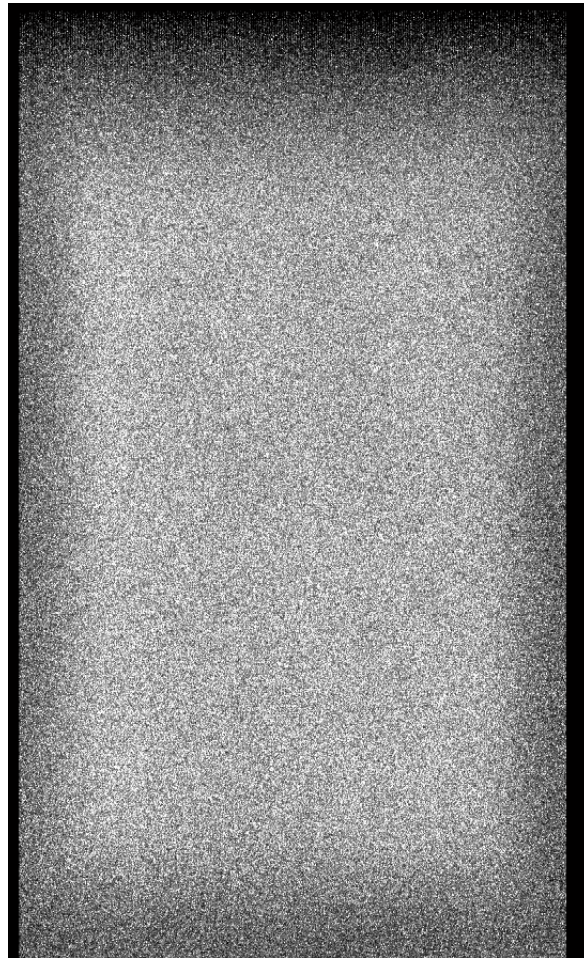


Figure 10.10: The dark current pattern in the MEM-FLIM3 camera.

10.3.3.3 Readout noise

The readout noise of the MEM-FLIM3 is shown in Table 10.4. The values of readout noise at different frequencies are not identical due to the varied configuration settings. The differences, however, are relatively small.

Table 10.4: The readout noise of the MEM-FLIM3 camera.

Unit	20 MHz	40 MHz	60 MHz	80 MHz	Mean
ADU	14.52	14.03	14.14	13.98	14.17
e-	54.60	54.79	55.88	53.58	54.71

10.3.4 Sensitivity

10.3.4.1 Sensitivity

The sensitivity of the MEM-FLIM3 camera is shown in Table. 10.5. We can see different regions have slightly different sensitivities and the sensitivity changes in a small range for different frequencies due to varied configurations. The average sensitivity for four regions at different frequencies is 0.26 ± 0.01 ADU/e-. Compared to the MEM-FLIM2 camera, the MEM-FLIM3 camera has a poorer sensitivity, a lower value of ADU/e-.

Table 10.5: The sensitivity (ADU/e-) of MEM-FLIM3 camera.

Region	Phase	20 MHz	40 MHz	60 MHz	80 MHz
A	one	0.266	0.253	0.262	0.267
[50:100,260:310]	two	0.266	0.253	0.240	0.258
B	one	0.254	0.256	0.249	0.259
[150:200,260:310]	two	0.266	0.253	0.248	0.256
C	one	0.269	0.259	0.263	0.263
[300:350,260:310]	two	0.272	0.251	0.255	0.263
D	one	0.264	0.260	0.247	0.256
[410:460,260:310]	two	0.270	0.265	0.259	0.264
Average		0.266	0.256	0.253	0.261
Standard Deviation		0.001	0.005	0.008	0.004

10.3.4.2 Detection limit

The minimum number of electrons that the MEM-FLIM3 can detect at different frequencies is shown in Table 10.6. The values are calculated based on the dark current measured in section 10.3.3.2 and the assumption that the noise floor is dominated by the readout noise when the integration time is short.

Table 10.6: The detection limit of MEM-FLIM3 camera.

Unit	20 MHz	40 MHz	60 MHz	40 MHz	Mean
e-	108.1	108.3	109.7	106.7	108.2

10.4 Lifetime measurement

10.4.1 System behavior and calibration

In this section, we focus on the imperfect performances of the MEM-FLIM3 camera in lifetime measurements. In the following cases, we measured the fluorescence lifetime for a yellow plastic slide and used a green fluorescence plastic ($\tau = 2.8$ ns) slide to calibrate the system. The fluorescence lifetime of the yellow plastic slide is measured by the reference camera to be between $\tau = 5.4$ ns to $\tau = 5.6$ ns. The MEM-FLIM3 camera is operated at 24 °C without Peltier cooling. The reasons to check the camera and system performance by using the fluorescence plastic slides are: (1) the plastic slides are not sensitive to photobleaching, (2) they contain a single lifetime component, (3) the lifetime across a slide is uniform.

10.4.1.1 Nonidentical column performance

We have noticed that the even and odd columns have different performances in generating intensity images and also lifetime images. The intensity and lifetime images have noticeable vertical stripes along the columns as shown in Fig. 10.11(a)(c)(e). The images were taken from a 50×50 region when the camera was operated at 20 MHz. For each column, the average intensity and lifetime values are calculated and plotted in Fig. 10.11(b)(d)(f), where systematic “oscillations” between even and odd columns are evident. The overall increasing trend of intensity values in Fig. 10.11(a) is not a major concern since it is caused by the non-uniform illumination. The differences in the lifetime values (Fig. 10.11(d)(f)), however, deteriorate the MEM-FLIM3’s performance. The phase and modulation information used to obtain lifetime values are shown in Fig. 10.12. The phase and modulation images are shown in Fig. 10.12(a) and (c), while their average values for each column are shown in Fig. 10.12(b) and (d), respectively. The oscillations between columns in the phase and modulation values lead to the systematic column difference in the lifetime values. There are, however, no systematic differences between even and odd columns in the linearity, sensitivity, dark current, etc.

We computed the lifetimes in the even and odd columns separately and compared them with the results when even and odd columns are considered without separation. The average values for each column are used for calculation in Table 10.7. The lifetime uncertainty (σ) measured in a region of interest is significantly larger when the even/odd column differences are not taken into consideration. As a result of the column differences, the lifetime uncertainty in an image consisting only even or odd columns can be

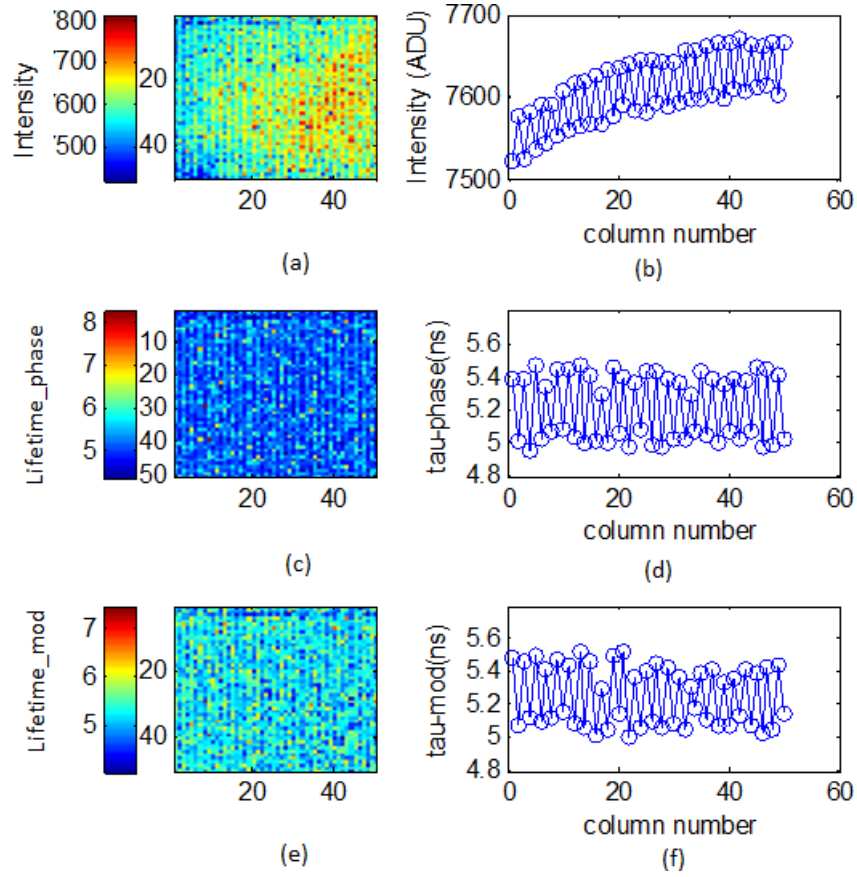


Figure 10.11: Column differences in intensity and lifetime image. (a) The intensity image, (b) the plot of the average intensity value of each column from (a), (c) the image of lifetime derived from the phase change, (d) the plot of the average lifetime value of each column from (c), (e) the image of lifetime derived from the modulation depth change, and (f) the plot of the average lifetime value of each column from (e).

approximately four times smaller than that of both columns taken together.

Table 10.7: The lifetime differences between columns in the MEM-FLIM3 camera.

Columns	lifetime-phase (ns)	lifetime-modulation (ns)
All	5.22 ± 0.19	5.26 ± 0.18
Odd	5.40 ± 0.05	5.43 ± 0.06
Even	5.03 ± 0.04	5.09 ± 0.04

In the pixel layout, the even and odd column pixels have slightly different designs regarding the positions of the metal contacts, as shown in Fig. 10.13. Two adjacent pixels

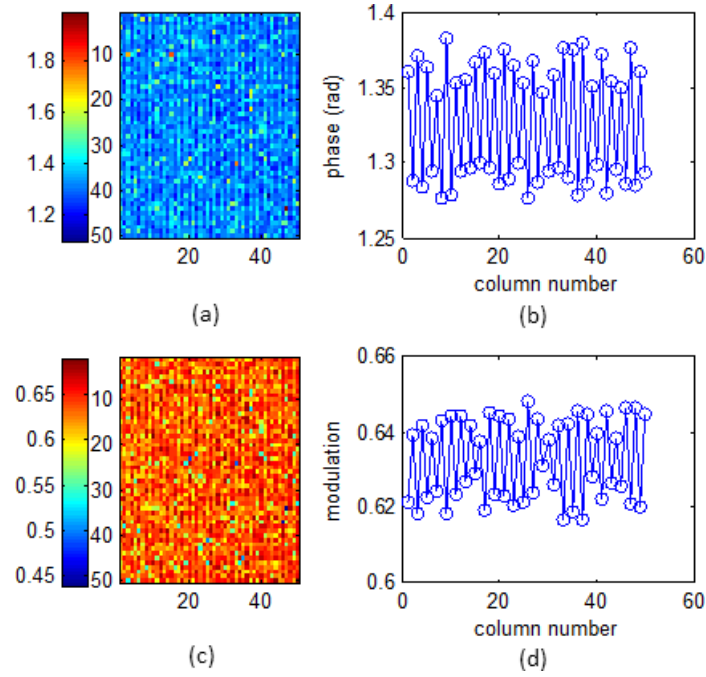


Figure 10.12: Column differences in phase and modulation. (a) The image of the phase information, (b) the plot of the average phase value of each column from (a), (c) the image of the modulation, (d) the plot of the average modulation value of each column from (c).

are shown in the green and red boxes, respectively. This unit is then horizontally repeated to form the whole image area. We suspect the differences in metal contacts in pixel layout might introduce this differential behavior between the odd and even columns.

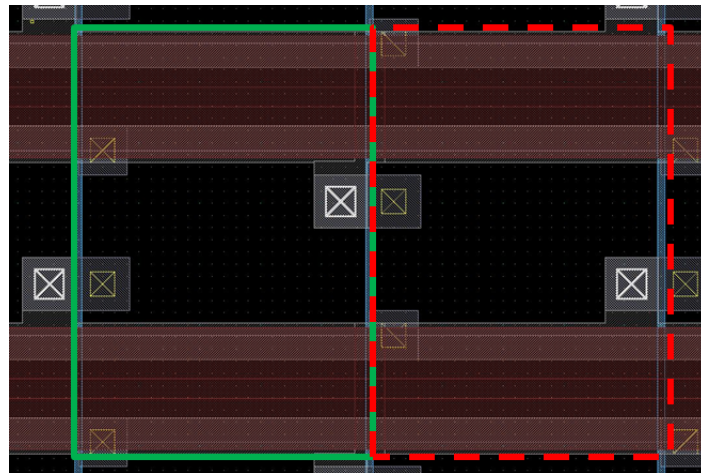


Figure 10.13: Metal contacts in pixel designs.

10.4.1.2 Nonidentical section performance

As we explained in Chapter 9, the image area of the MEM-FLIM3 sensor has been divided into four sections in the vertical directions as shown in Fig. 9.1. Due to the identical designs, we expect the same response across each of these sections. We have evaluated and concluded that the four sections have approximately the same linearity, sensitivity, etc. The separations, however, have also introduced some artifacts.

For certain settings of the MEM-FLIM3 camera (for example, the configurations at 40 MHz), we can see a clear separation between the four image sections both from the intensity image and the lifetime images, as shown in Fig. 10.14. If we plot the intensity along two columns (one even column and one odd column) through four sections, we can see the sudden change of the intensity between different sections, as shown in Fig. 10.15. In Fig. 10.15, not only the difference in the even and odd column is presented by the red and dark blue curves, but also the difference in two phases by the green and light blue curves. The section difference in the intensity image is a drawback for end users due to the visual effect. Furthermore, the section difference in the lifetime images is not acceptable. We took small regions of interest in each of the four sections and listed the lifetime values in Table 10.8. There is approximately 0.3 ns difference in lifetime between section 1 and section 4. The lifetimes measured from the reference camera are 5.42 ± 0.25 ns (lifetime from the phase) and 5.53 ± 0.18 ns (lifetime from the modulation).

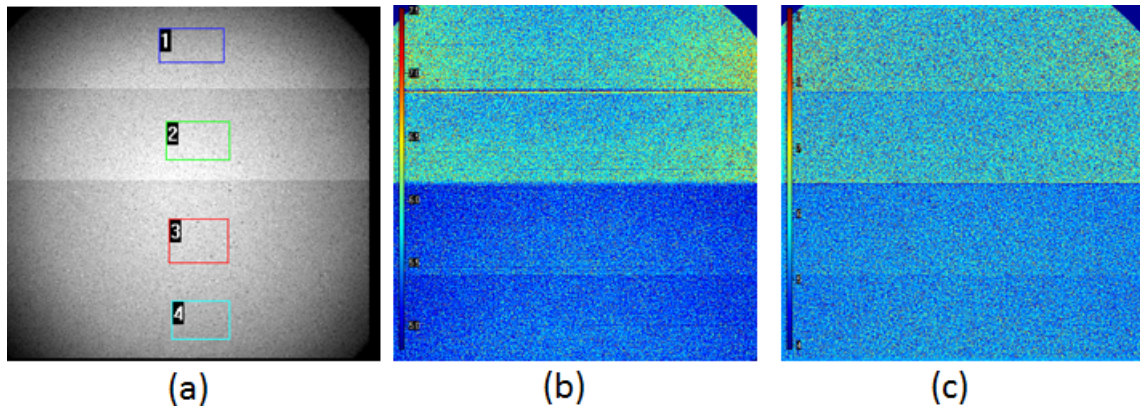


Figure 10.14: Section differences in intensity and lifetime image. (a) The intensity image, (b) the image of lifetime derived from the phase change and (c) the image of lifetime derived from the modulation depth change.

The intensity difference does not necessarily lead to the lifetime difference. In cases of nonuniform illumination or different fluorophore concentrations in the single lifetime component sample, the intensity values are different at different parts of the image. The lifetime values, however, are uniform. This is the main advantage of fluorescence lifetime which biologists favor: its independence from the fluorescence intensity. In our case, the differences between different sections in the lifetime image is caused by the four sections reacting differently to different phase delays which we applied between the LED light and

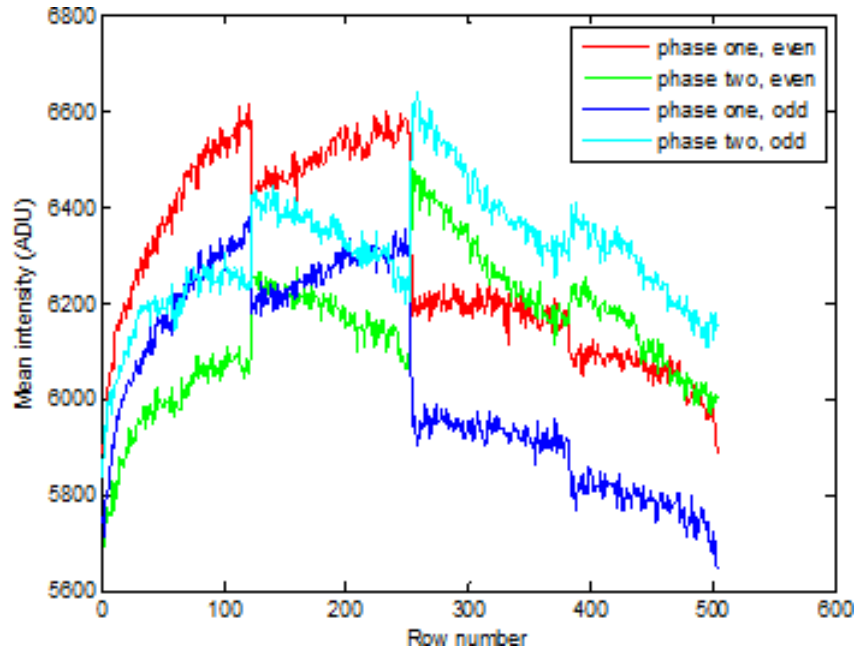


Figure 10.15: Intensity plot along a column in the MEM-FLIM3 camera at 40 MHz.

Table 10.8: The lifetime differences accross different sections in the MEM-FLIM3 camera.

Section number	lifetime-phase (ns)	lifetime-modulation (ns)
1	5.81 ± 0.49	5.87 ± 1.72
2	5.83 ± 1.15	5.78 ± 1.26
3	5.49 ± 0.48	5.49 ± 0.96
4	5.47 ± 0.44	5.48 ± 1.04

the demodulation signal on the toggle gates of the camera. In the ideal case, the four sections should react the same at different phase delays, as shown in Fig. 10.16(a). This figure shows the intensity plot along a column (column number 400) at different phase delays when the MEM-FLIM3 camera was operated at 20 MHz. The horizontal axis is the row number along the column, and the vertical axis is the intensity value (ADU). Since every image contains two phase images, when plotting the intensity along the column, one will see the intensities of the two phase images. At some phase delay, the intensity differences between two phase images are small, which leads to a narrower band in the plotting as shown in the topleft image. Big differences between two phase images at other phase delays give a wider band as shown in the bottom right image in Fig. 10.16(a). From the plot we can see the shading due to the non-uniform illumination, but the connections are smooth between four sections. The four sections react in the same way through all

the phase delays. When the camera is operated at 40 MHz, however, the four sections react differently, as shown in Fig. 10.16(b). We can see clear separations between the first three sections, these differences also affect the lifetime values. The third and fourth sections have a similar response and yield close lifetime values.

10.4.1.3 Total intensity calibration

Phenomenon

When changing the phase delay between the LED light source and the demodulation signals which are applied on the toggle gates, we can measure two modulation curves for each pixel from its two phase registers. These are shown as “phase one” and “phase two” in Fig. 10.17. We can see at 20, and 40 MHz, the modulation curves are reasonably good. The modulation curves are sine waves instead of square waves due to the fact that the LED light shape is closer to a sine wave. The curves at 60, and 80 MHz, however, are distorted.

In an ideal situation, the sum of the charges in two phase registers from one pixel remain the same through different phase delays between the light source and the camera demodulation, while the distribution of the charge between the two phase register changes. After adding up the charges from the two phase registers, we found that the total intensity from two phase registers of one pixel did not remain the same, as shown in Fig. 10.18. There were 4%, 17%, 53%, and 77% change in the intensity when the camera was operated at 20, 40, 60, and 80 MHz, respectively. The measurements are done over a 50×50 region.

Causes

In order to find the cause of this bad modulation behavior, we checked (1) the camera toggle gate demodulation signal and LED driver signal, as shown in Fig. 10.19, and (2) the LED light output signal, as shown in Fig. 10.20.

In Fig. 10.19, the yellow curve is the camera output signal which is used to drive the LED. The shapes of the LED driver signal are close to a square wave at all frequencies. The demodulation signals (green curve) at four frequencies are generated in the same way, they start at the timing generator as a square wave but the camera electronics and the sensor arrangement alter the shape of the signal in a way that is difficult to predict. The higher the frequency, the bigger the distortion in the demodulation signal. At 80 MHz, the demodulation signal is no longer symmetrical. This is not desired for the lifetime measurement. The light output of the LED is shown in Fig. 10.20. Compared to the LED signals at higher frequencies, the LED signal at 20 MHz has higher frequencies and looks more like a square wave.

The width of the LED signal has a slight change (400 ps) throughout different phase delays at 80 MHz when the duty cycle of the LED is set to 50%. This $0.4\text{ns}/(12.5\text{ns} * 50\%) = 8\%$ width change of the light source not only affects the accuracy of the lifetime, but also has a significant influence on the power of the light output. We measured the power of the LED light at the exit of the objective (Zeiss air objective with a magnification

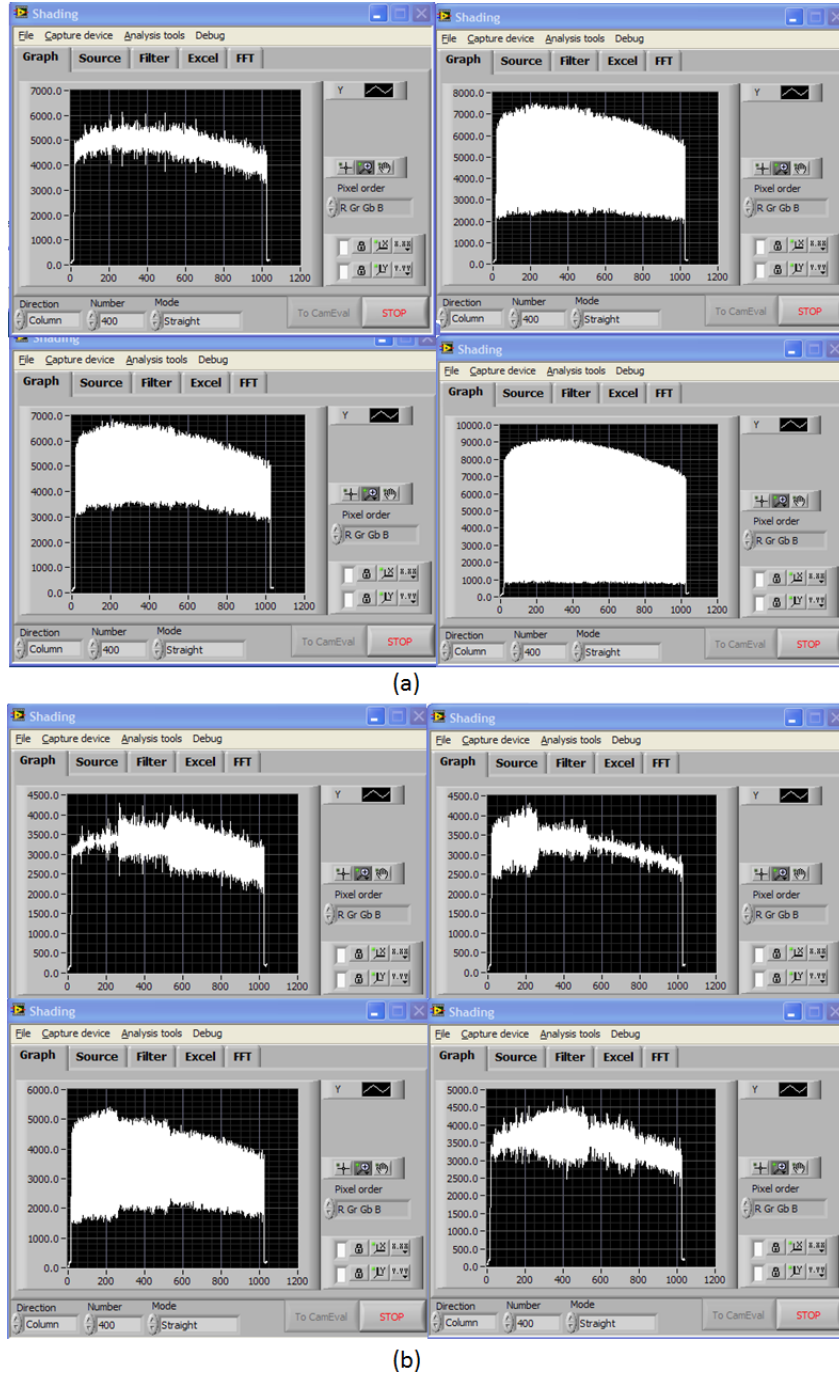


Figure 10.16: Column intensity plot through different phase delays. (a) A uniform reaction between the four sections, and (b) a non-uniform reaction between the four sections.

of $20\times$ and a numerical aperture $NA = 0.5$), as shown in Fig. 10.21. When the LED is controlled by the MEM-FLIM3 camera, we can see the power of the light source has

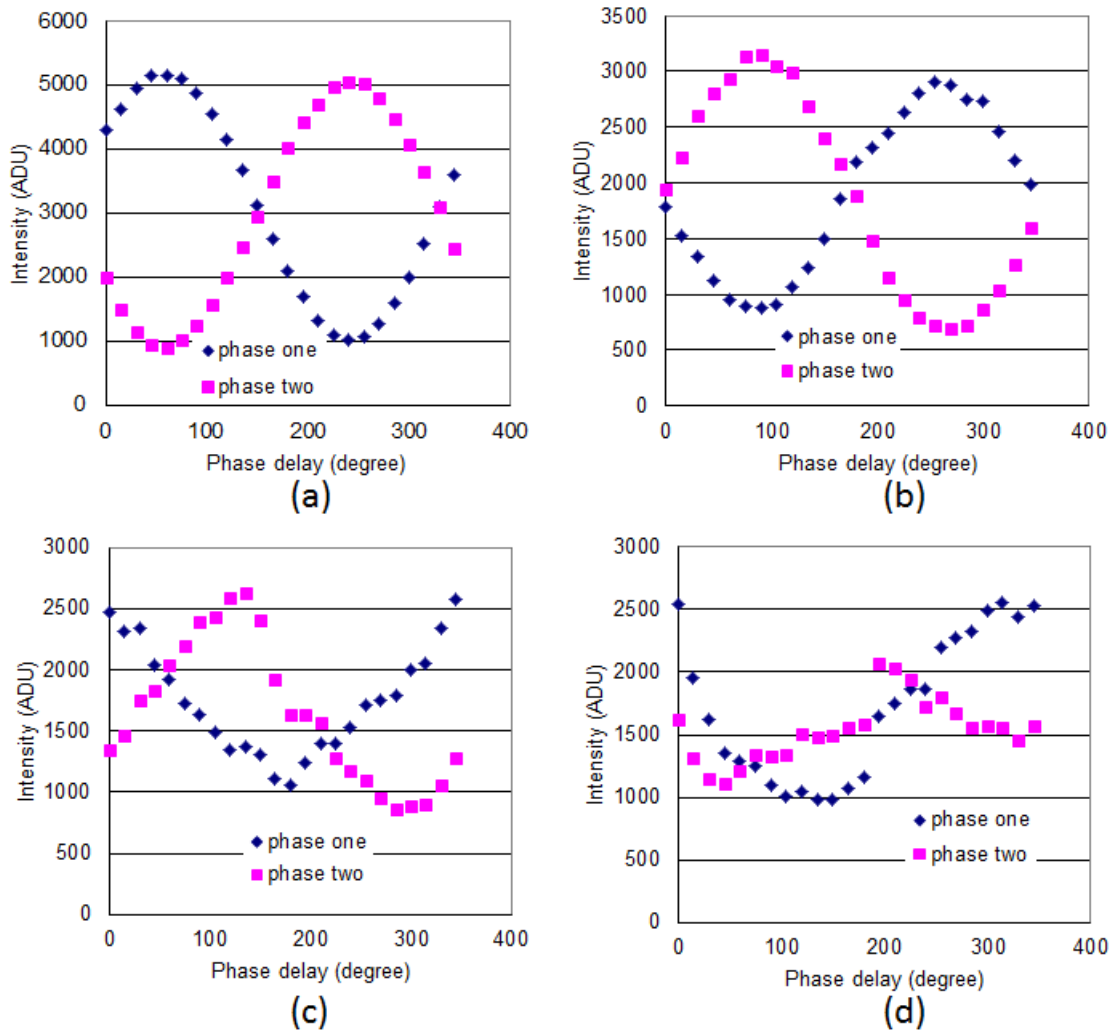


Figure 10.17: Modulation curve before intensity correction. The camera is modulated at (a) 20MHz, (b) 40MHz, (c) 60MHz, and (a) 80MHz.

a very big change, in this case a 133% change. The shape of the light power resembles the shape of the sum intensity in Fig. 10.18(d). We conclude the slight change in the width of the LED driver signal from the MEM-FLIM3 camera leads to a considerable power output change from the LED, which results in the differences of the total intensity of the two phase registers through the various phase delays. This leads to a distorted modulation curve.

Calibration

Instead of driving the LED directly from the MEM-FLIM3 camera, one can use an external pulse generator to obtain a more stable signal to drive the LED. When the LED

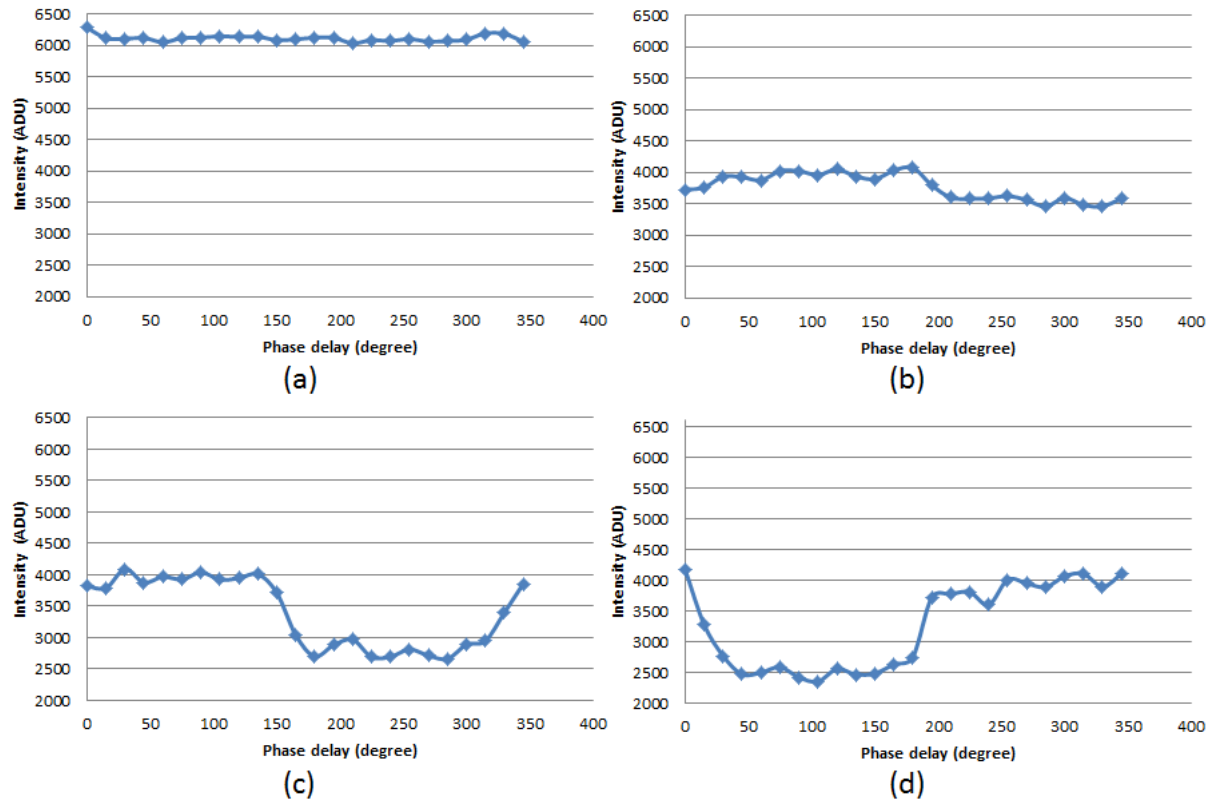


Figure 10.18: The sum of two phase register measurements. The camera is modulated at (a) 20MHz, (b) 40MHz, (c) 60MHz, and (a) 80MHz.

is controlled by the external pulse generator, the power curve is relatively stable (with 14% change). The fluctuation in the LED intensity can be avoided in this way, and a better modulation curve can be obtained, as shown in Fig. 10.22. In this case there is only 1% change of the total intensity from the sum of the two phase registers.

The data obtained when controlling the LED directly by the MEM-FLIM3 camera can be corrected by normalizing the total intensity from the two phase registers at each phase delay. The resulting modulation curve after correction is shown in Fig. 10.23. This correction eliminates the need for an external pulse generator and can keep the system compact.

10.4.1.4 DC shift calibration

Phenomenon

When the MEM-FLIM3 camera is illuminated with a constant light source (instead of the modulated one), the two phase registers of one pixel should in the ideal situation separate the charge equally, as shown in Fig. 10.24(a). The x axis is the total intensity

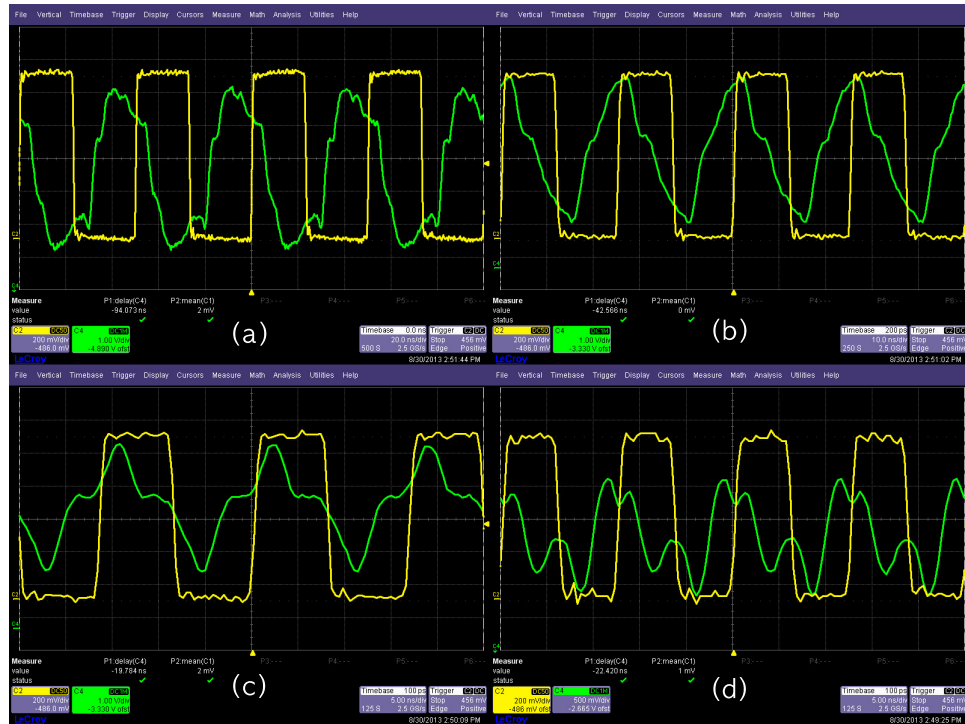


Figure 10.19: The camera toggle gate demodulation signal (green) and the LED driver signal (yellow). The camera is modulated at (a) 20MHz, (b) 40MHz, (c) 60MHz, and (a) 80MHz.

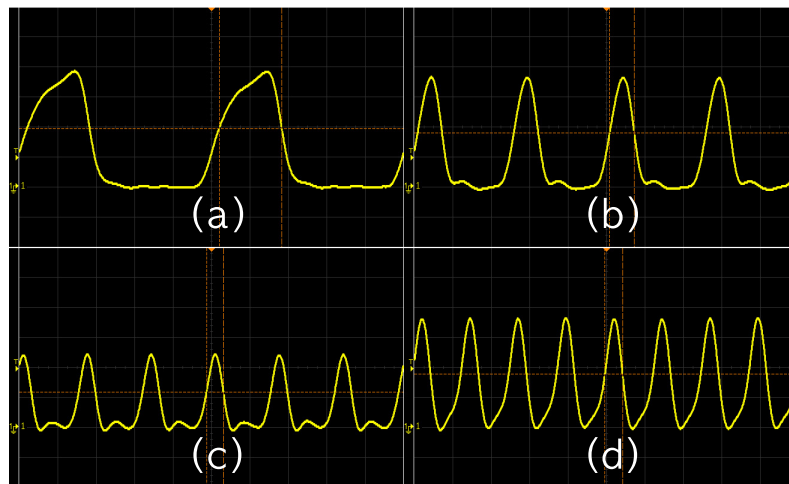


Figure 10.20: The LED output signal. The camera is modulated at (a) 20MHz, (b) 40MHz, (c) 60MHz, and (a) 80MHz.

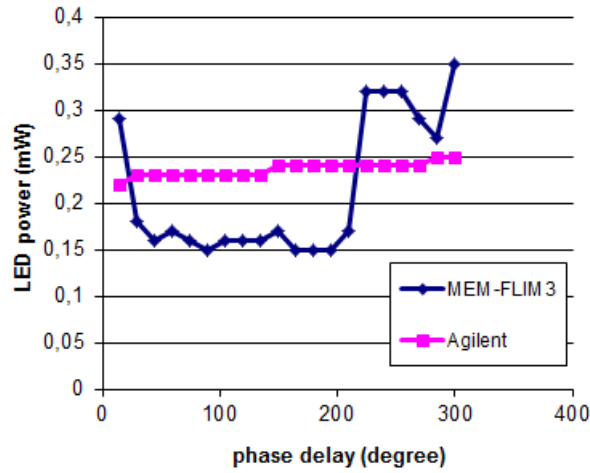


Figure 10.21: The power of the LED signal. The LED is controlled either by the MEM-FLIM3 camera or an external pulse generator.

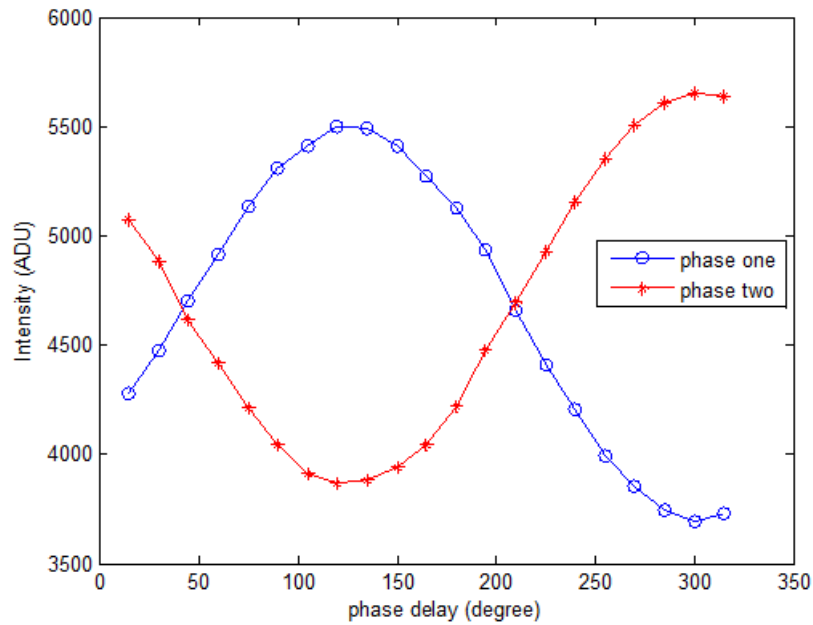


Figure 10.22: The modulation curve when the LED driver signal is controlled by an external pulse generator.

of the two phase registers from one pixel at different illumination intensities, the y axis is the intensity from each phase register. The slopes of the two curves are both close to

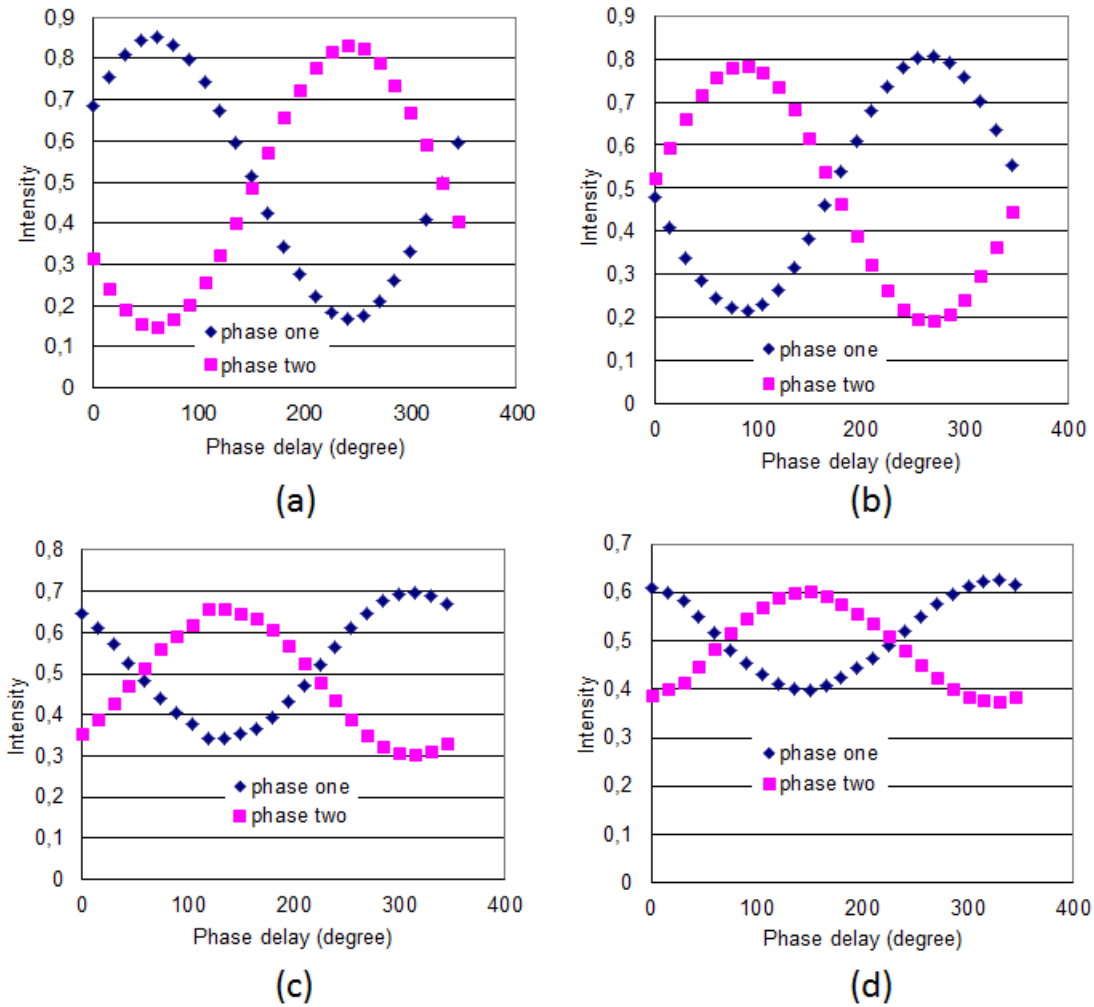


Figure 10.23: Modulation curve after intensity correction. The camera is modulated at (a) 20MHz, (b) 40MHz, (c) 60MHz, and (a) 80MHz.

50%, meaning that they are splitting the charge equally. This is, however, not valid for all pixels. For example, in Fig. 10.24(b), the two phase registers have different abilities in separating charges. One phase register collects 59% of the total charge while the other collects just 41%. There is a preference for the charges to go into one of the two phase registers.

This preference for the charges to go into one phase register leads to a significant DC shift between two phase registers when the camera and light source are both modulated at 80 MHz. We then see a gap between the first half and the second half of the modulation curve for those pixels which do not separate charge equally between two phase registers when illuminated by a constant light source. The sudden change occurs in the middle of the modulation curve due to the fact we use the charges collected by one phase register

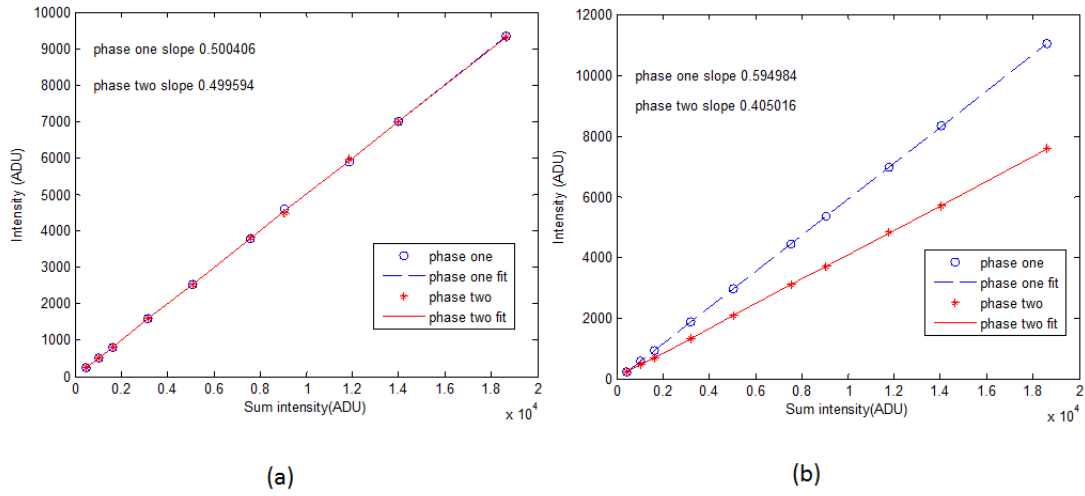


Figure 10.24: Charge separation (a) ideal case, and (b) nonideal case.

in the first half of the modulation curve, while the second half of the modulation curve is collected by the other phase register. Figure 10.25(a) shows a pixel which generates continuous modulation curves while Fig. 10.25(b,c) shows discontinuous modulation curves. The dots are the experimental data, and lines are the fitted curves. We can see that the fitting is clearly incorrect when there is a gap between the first and second half of the phase information. The two colors in the images represent two experiments: one curve is green plastic slide data which is used to calibrate the system (blue), the other curve (red) is the yellow plastic slide data. Only 0.67% of the pixels have modulation curves which are continuous at 80 MHz. The higher the modulation frequency is, the fewer pixels from which we get continuous modulation curves. This phenomenon is only well pronounced when the camera is modulated at 80 MHz.

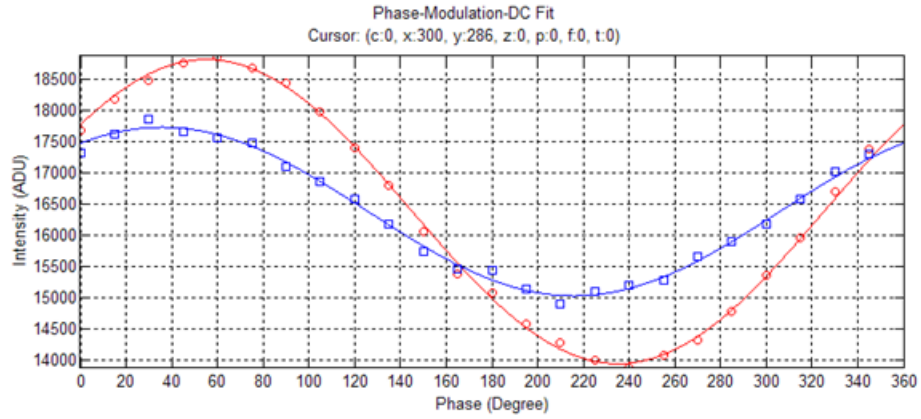
Causes

The preference of one phase register above the other is caused by the non-symmetrical potential profiles along one pixel. A slight difference in the potential causes one phase register to receive more charge than the other. This can be influenced by the voltages applied on the toggle gates, or by the fabrication process of the sensor.

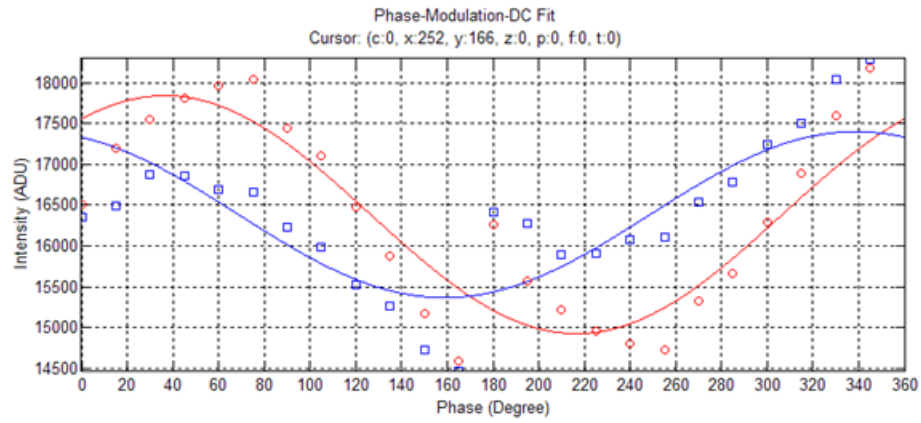
Calibration

Optimizing the DC voltages of the toggle gate can minimize this unequal charge splitting ability. This, however, cannot be done at the pixel level.

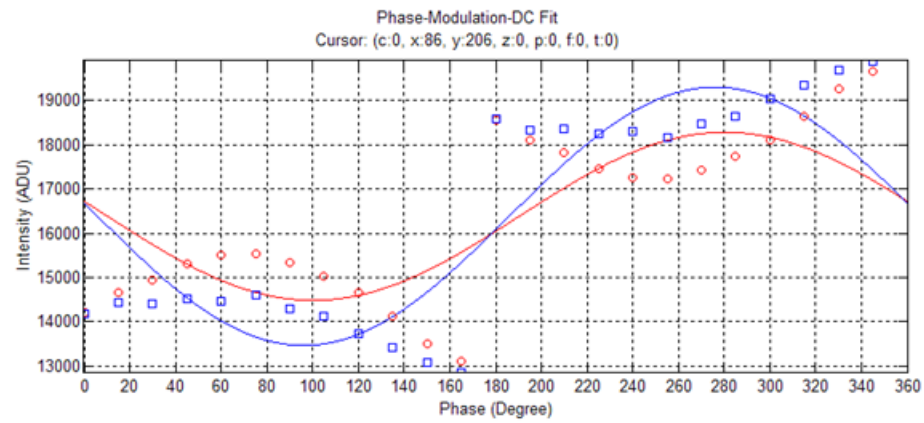
In order to get valid lifetime values, the calibration has to be done at the pixel level. When changing the phase delay between the light source and demodulation signal by every 15 degrees, we obtain 24 images from the MEM-FLIM3 camera, each of which contains



(a)



(b)



(c)

Figure 10.25: Modulation curve: (a) ideal case, and (b) (c) nonideal case.

two phase measurements. When adding the intensity from one phase, we average out the AC components in the sine curve and were able to get the DC values. We can then use these DC values to calibrate the difference between the two phase registers to get rid of the discontinuous (jump) in the modulation curve. After the calibration, all the pixels have smooth modulation curves.

10.4.2 Lifetime examples

10.4.2.1 Plastic slide

The lifetimes of the yellow fluorescence plastic slide measured by the MEM-FLIM3 camera and the reference camera are shown in Table 10.9. A Zeiss objective with a magnification of $20\times$ and a numerical aperture of 0.5 has been used. The integration times from both the cameras were set to 40 ms. The MCP voltage for the reference camera was set to 400V. The green plastic slide with the lifetime of 2.8 ns was used to calibrate the system in order to get the phase change and modulation depth change introduced by the system (see Chapter 7). For the reference camera, 24 images are taken with a phase step of 15 degrees. For the MEM-FLIM3 camera, 12 images with the same phase step (15 degrees) are used for the lifetime measurement since every image consists of two phase images.

For 20 and 40 MHz, the MEM-FLIM3 achieves reasonable lifetime values compared with the reference camera. The lifetime uncertainties (σ) from the MEM-FLIM3 camera were, however, higher than those from the reference camera. The higher lifetime uncertainties are caused by the higher noise source (dark current and readout noise).

For 20 and 40 MHz, the images were used directly to obtain lifetime values. For 60 and 80 MHz, the image data went through two calibration steps before the lifetimes were calculated: (1) to get rid of the total intensity difference for different phase steps, as explained in Section 10.4.1.3, and (2) to get rid of the DC difference between two phase information, as explained in Section 10.4.1.4. After corrections, the lifetimes derived from the modulation depth change are in reasonable ranges. The lifetimes from the phase change, however, are far from the lifetimes measured by the reference camera.

Instead of carrying out a second step of the calibration, we could also use the information from only one phase for the MEM-FLIM3 camera. The MEM-FLIM3 camera then functions in same way as the reference camera, and in total 24 images are needed instead of 12 images. The lifetimes obtained in this method from the MEM-FLIM3 camera are comparable with the ones from the reference camera. This means despite the unequal abilities for splitting charge between two registers, the demodulation for either register works reasonably well and the information stored in one register can be used to retrieve the fluorescence lifetime.

10.4.2.2 GFP labeling fixed U2OS cells

The lifetime measurements were performed on the GFP labeling U2OS cells. A Zeiss oil objective with a magnification of $40\times$ and a numerical aperture of 1.3 was used for these

Table 10.9: The lifetimes of the fluorescence plastic slides.

Freq (MHz)	Camera	τ_θ (ns)	τ_m (ns)
20	Reference	5.48 ± 0.13	5.59 ± 0.19
	MEM-FLIM3	5.45 ± 0.65	5.54 ± 0.52
40	Reference	5.42 ± 0.24	5.53 ± 0.18
	MEM-FLIM3	5.47 ± 0.47	5.56 ± 0.91
60	Reference	5.49 ± 0.51	5.51 ± 0.24
	MEM-FLIM3	1.59 ± 0.14	5.25 ± 0.46
	MEM-FLIM3(single phase)	5.44 ± 0.56	5.48 ± 0.66
80	Reference	5.43 ± 1.12	5.51 ± 0.39
	MEM-FLIM3	1.65 ± 0.25	5.69 ± 0.86
	MEM-FLIM3(single phase)	5.64 ± 1.01	5.86 ± 1.18

experiments. The LED was controlled by the MEM-FLIM3 camera directly. We have used a $10 \mu\text{M}$ fluorescein solution (Sigma Aldrich 46955) ($\tau = 4 \text{ ns}$) [136, 137] for the system calibration. The same gray value stretching processes as described in Section. 8.4.1 were applied to the intensity images. The results of measurements are presented in Table 10.10. The lifetimes from the reference camera are different at four frequencies since different cells were measured at different frequencies. The difference between the lifetimes derived from the phase change and the modulation change can be explained by the heterogeneity of GFP lifetime components, as explained in the MEM-FLIM2 evaluation results in Chapter 8. The results from the MEM-FLIM3 camera at 20 and 40 MHz are comparable with the ones from the reference camera. The lifetimes measured by the MEM-FLIM3 camera, however, have a higher uncertainty than the ones from the reference camera. The lifetimes derived from the modulation depth change from the MEM-FLIM3 camera at 60 and 80 frequencies are also in an acceptable range. The lifetime derived from the phase change cannot be trusted. The MEM-FLIM3 can also be operated in the same way as the reference camera using phase information from only one register. The lifetimes from the phase obtained in this way can be compared with those from the reference camera. From the images at 20 MHz (Fig. 10.26) and 40 MHz (Fig. 10.27), we can see that the MEM-FLIM3 camera has a higher resolution and a better image quality than the reference camera. In Fig. 10.28, both cameras were modulated at 80 MHz. Intensity images from the reference cameras with a lower MCP voltage (Fig. 10.28(b)) and a higher MCP voltage (Fig. 10.28(c)) are compared with one from the MEM-FLIM3 camera (Fig. 10.28(a)) at the same integration time (800ms). The MEM-FLIM3 camera generates a better image with lower noise compared to the reference camera.

Table 10.10: The lifetimes of the GFP labeling fixed U2OS cells.

Freq (MHz)	Camera	Integration(ms)[MCP(V)]	τ_θ (ns)	τ_m (ns)
20	Reference	400[600]	3.11 ± 0.19	4.15 ± 0.30
	MEM-FLIM3	400	3.18 ± 0.89	4.65 ± 1.27
40	Reference	500[600]	2.44 ± 0.17	3.73 ± 0.46
	MEM-FLIM3	500	2.46 ± 0.23	3.64 ± 0.59
60	Reference	800[600]	1.89 ± 0.11	2.98 ± 0.11
	MEM-FLIM3	800	7.79 ± 66	2.76 ± 1.06
80	Reference	200[700]	1.36 ± 0.49	2.36 ± 0.56
	MEM-FLIM3	800	$-12.43.46 \pm 123$	2.75 ± 0.84
	M3 single phase	800	1.02 ± 0.21	4.22 ± 1.77

10.5 Conclusion

The comparison between the MEM-FLIM2, MEM-FLIM3, and reference cameras are shown in the Table 10.11. To simplify the comparison, the values of the MEM-FLIM3 camera shown in the table are the average performances at four frequencies. The MEM-FLIM3 camera has proper masks and no misalignment for the shielding, thus the mask problem that appeared in the MEM-FLIM1 and MEM-FLIM2 cameras has been eliminated in the MEM-FLIM3 camera. Compared to the MEM-FLIM2 camera, the advantage of the MEM-FLIM3 camera is the ability to measure lifetimes at higher frequencies. The performances of sensitivity, dark current, and readout out noise, however, are not as good as the MEM-FLIM2 camera due to the complex camera and sensor design. Camera electronics and sensor performance could be improved by camera redesign and wafer processing optimisation.

The lifetimes measured by the MEM-FLIM3 camera are comparable with the ones from the reference camera at lower frequencies (20, 40 MHz) with slightly higher lifetime uncertainties. The images obtained by the MEM-FLIM3 camera have a better resolution when imaging biological samples. There are, however, column differences (20MHz) and section differences (40 MHz) in the intensity and lifetime images. For higher frequencies (60, 80 MHz), images obtained from the MEM-FLIM3 camera need calibration in order to be used for lifetime calculation. The lifetimes derived from the modulation depth change are in an acceptable range when using two phase register information. The lifetime derived from the phase, however, is not reliable. The lifetimes derived from the phase by only using one phase register from the MEM-FLIM3 camera are comparable with the ones from the reference camera.

At the end of the MEM-FLIM project, a four-wavelength LED light source (446, 469,

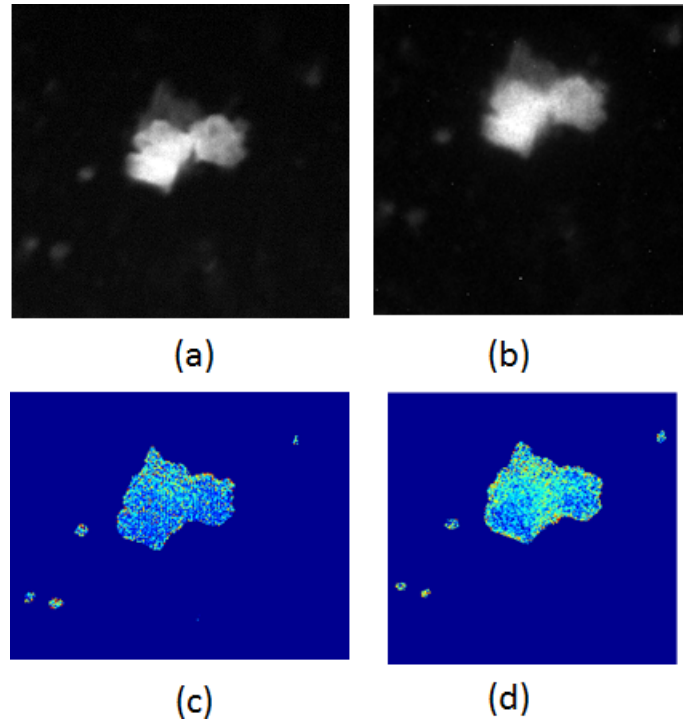


Figure 10.26: Lifetimes for GFP labeling fixed U2OS cells at 20 MHz. (a) and (b) are the intensity images from the MEM-FLIM3 camera and the reference camera, respectively. (c) and (d) are the lifetime images from the MEM-FLIM3 camera and the reference camera, respectively.

Table 10.11: Performance comparison of the MEM-FLIM2, MEM-FLIM3 and the reference cameras.

	MEM-FLIM2	MEM-FLIM3	Reference
Sampling density (samples/ μm @ $20\times$)	1.24×1.24	0.9×0.9	1.07×1.07
OTF @ 500 cycles/mm	0.75	0.54	0.39
Sensitivity(ADU/ e^-)	0.43 ± 0.03	0.26 ± 0.01	0.53 ± 0.03
Detection limit at short integration time(e^-)	51.4	108.2	35.4
Linearity	0.999995	0.999905	0.999385
$\sigma_{readout}$ ADU(e^-)	5.9(13.72)	14.16(54.71)	3.4(5.67)
Dark current (e^-/ms)	0.29	1.87	0.08

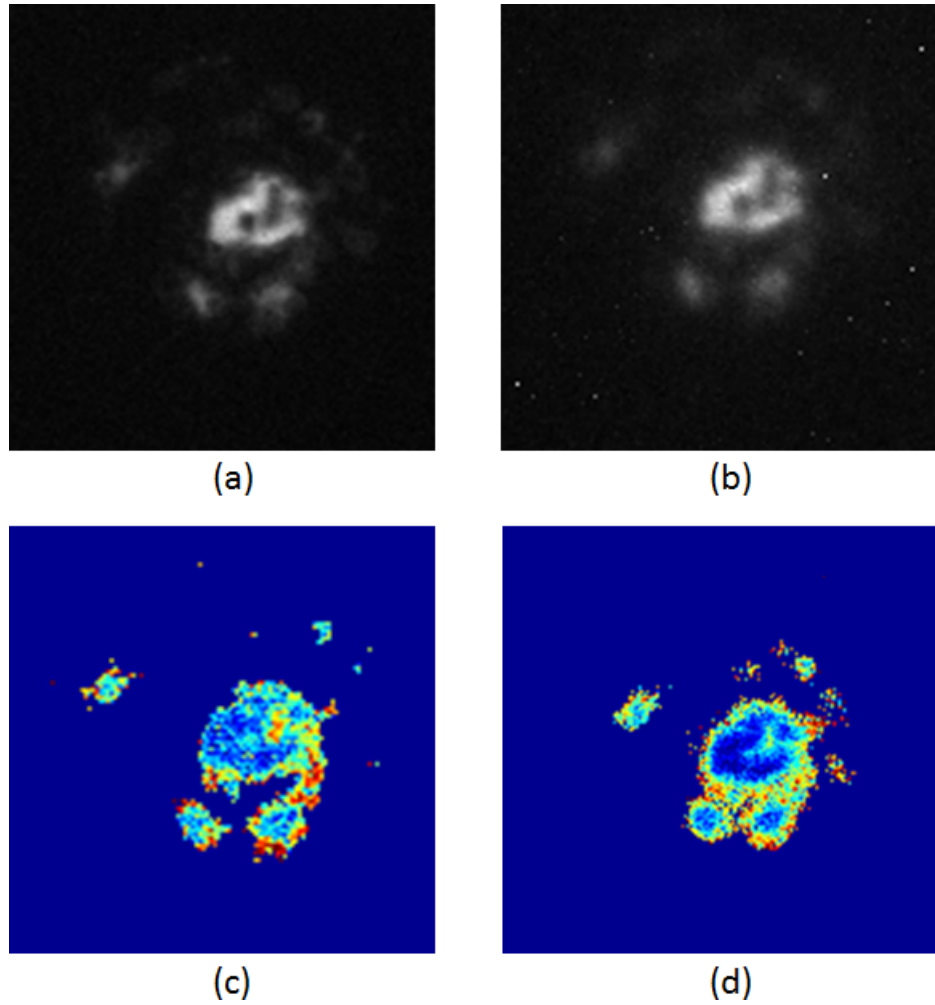


Figure 10.27: Lifetimes for GFP labeling fixed U2OS cells at 40 MHz. (a) and (b) are the intensity images from the MEM-FLIM3 camera and the reference camera, respectively. (C) and (d) are the lifetime images from the MEM-FLIM3 camera and the reference camera, respectively.

523, 597 nm) has been built, and the MEM-FLIM3 camera has been put into a proper camera housing by Lambert Instruments, as shown in Fig. 10.29. The MEM-FLIM3 camera can be operated without fans and without Peltier cooling eliminating a potential source of vibration and additional electronics. The sensor temperature remains below 50 °C even when it is modulated at 80 MHz for several hours. This setup has been installed in the Cell Biophysics and Imaging group in the Netherlands Cancer Institute for further experiments and evaluations.

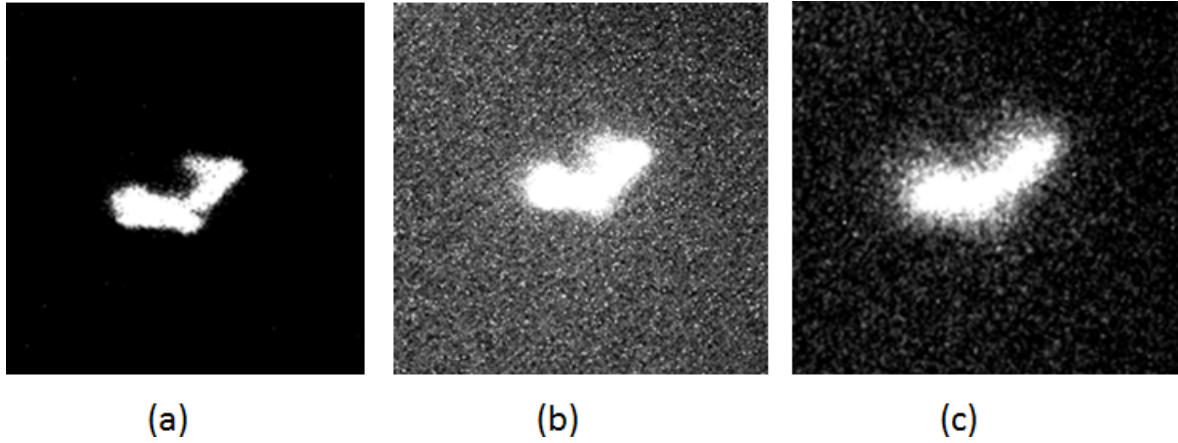


Figure 10.28: Intensity images for GFP labeling fixed U2OS cells at 80 MHz. (a) the MEM-FLIM3 camera, (b) the reference camera with MCP at 500 V, and (c) the reference camera with MCP at 700 V.

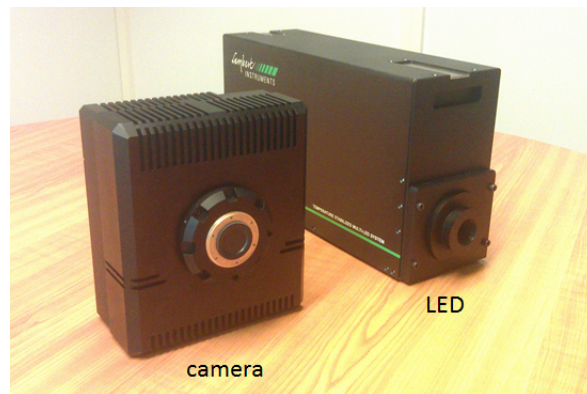


Figure 10.29: The MEM-FLIM3 camera in a proper camera housing and the multi-wavelength LED.

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A Solid-State Camera System for Fluorescence Lifetime Microscopy

- Qiaole Zhao -

Fluorescence microscopy is a well-established platform for biology and biomedical research (Chapter 2). Based on this platform, fluorescence lifetime imaging microscopy (FLIM) has been developed to measure fluorescence lifetimes, which are independent of fluorophore concentration and excitation intensity and offer more information about the physical and chemical environment of the fluorophore (Chapter 3). The frequency domain FLIM technique offers fast acquisition times required for dynamic processes at the sub-cellular level. A conventional frequency-domain FLIM system employs a CCD camera and an image intensifier, the gain of which is modulated at the same frequency as the light source with a controlled phase shift (time delay). At the moment these systems, based on modulated image intensifiers, have disadvantages such as high cost, low image quality (distortions, low resolution), low quantum efficiency, prone to damage by overexposure, and require high voltage sources and RF amplifiers. These disadvantages complicate the visualization of small sub-cellular organelles that could provide valuable fundamental information concerning several human diseases (Chapter 3 and 4).

In order to characterize the constraints involved in current fluorescent microscope systems that are used for lifetime as well as intensity measurements and to design and fabricate new systems, we have constructed a mathematical model to analyze the photon efficiency of frequency-domain fluorescence lifetime imaging microscopy (FLIM) (Chapter 5). The power of the light source needed for illumination in a FLIM system and the signal-to-noise ratio (SNR) of the detector have led us to a photon “budget”. A light source of only a few milliWatts is sufficient for a FLIM system using fluorescein as an example. For every 100 photons emitted, around one photon will be converted to a photoelectron, leading to an estimate for the ideal SNR for one fluorescein molecule in an image as 5 (14 dB). We have performed experiments to validate the parameters and assumptions used in the mathematical model. The transmission efficiencies of the lenses, filters, and mirrors in the optical chain can be treated as constant parameters. The Beer-Lambert

law is applicable to obtain the absorption factor in the mathematical model. The Poisson distribution assumption used in deducing the SNR is also valid.

We have built compact FLIM systems based on new designs of CCD image sensors that can be modulated at the pixel level. Two different designs: the horizontal toggled MEM-FLIM1 camera and vertical toggled MEM-FLIM2 camera are introduced (Chapter 6). By using the camera evaluation techniques described in Chapter 7, these two versions of the MEM-FLIM systems are extensively studied and compared to the conventional image intensifier based FLIM system (Chapter 8). The low vertical charge transport efficiency limited the MEM-FLIM1 camera to perform lifetime experiments, however, the MEM-FLIM2 camera is a success. The MEM-FLIM2 camera not only gives comparable lifetime results with the reference intensifier based camera, but also shows a much better image quality and reveals more detailed structures in the biological samples. The novel MEM-FLIM systems are able to shorten the acquisition time since they allow recording of two phase images at once.

The MEM-FLIM2 camera is, however, not perfect. It can only be modulated at a single frequency (25 MHz) and requires that the light source be switched off during readout due to an aluminum mask that had a smaller area than intended. A redesign of the architecture based on the vertical toggling concept leads to the MEM-FLIM3 camera (Chapter 9). Several improvements have been made in the sensor design for the MEM-FLIM3 camera, such as higher fill factor, greater number of pixels etc. The MEM-FLIM3 camera is able to operate at higher frequencies (40, 60 and 80 MHz) and has an option for electron multiplication. Evaluations of this updated MEM-FLIM system are presented (Chapter 10). The images obtained from the MEM-FLIM3 camera at 20 and 40 MHz can be used directly for the lifetime calculation and the obtained lifetimes are comparable with the ones from the reference camera. There are, however, differences in the even and odd columns (20 MHz) and four image sections (40 MHz) for the intensity and lifetime images. For higher frequencies (60 and 80 MHz) calibrations are needed before calculating lifetimes. The lifetimes derived from the modulation depth after the calibrations are in a reasonable range while the lifetime derived from the phase cannot be used. At 60 and 80 MHz we can use one phase register from the MEM-FLIM3 camera for the lifetime calculation, the same way the reference camera operates. The lifetimes obtained by this method from the MEM-FLIM3 at 60 and 80 MHz are comparable with the ones from the reference camera. The MEM-FLIM3 camera also has an electron multiplication feature for low-light experimental condition. We could get approximately 500 times multiplication. Lifetime measurement using the EM function, however, has not been tested due to the limitation of the project time.

Modulated All Solid-State Camera based Fluorescence Lifetime Imaging Microscopy

- Qiaole Zhao -

Fluorescentiemicroscopie is een gerenommeerd platform voor biologie en biomedisch onderzoek (hoofdstuk 2). Op basis van dit platform is Fluorescence Lifetime Imaging Microscopy (FLIM) ontwikkeld om de fluorescentie levensduren, die onafhankelijk zijn van fluorofoor concentratie en excitatie-intensiteit, te meten. Deze bieden meer informatie over de fysieke en chemische omgeving van de fluorofoor (hoofdstuk 3). De frequentiedomein gebaseerde FLIM techniek maakt snelle acquisitietijden mogelijk die nodig zijn voor het meten van dynamische processen op sub-cellulair niveau. Een conventioneel frequentiedomein gebaseerd FLIM systeem gebruikt een CCD camera en een beeldversterker. De versterking van deze beeldversterker wordt gemoduleerd met dezelfde frequentie als die van de lichtbron, echter met een gecontroleerde faseverschuiving (vertraging). De huidige systemen die gebaseerd zijn op gemoduleerde beeldversterkers hebben nadelen zoals hoge kosten, lage beeldkwaliteit (geometrische vervormingen, lage resolutie), lage kwantumefficiëntie, gevoelig voor beschadiging door overbelichting, vereisen hoge stuurspanningen en noodzakelijke RF-versterkers. Deze nadelen bemoeilijken de visualisatie van kleine sub-cellulaire organellen die waardevolle fundamentele informatie verschaffen over menselijke ziekten (hoofdstuk 3 en 4).

Om de beperkingen te karakteriseren van de huidige fluorescentiemicroscoop die gebruikt worden voor de levensduur en intensiteitsmetingen en het daarmee mogelijk te maken nieuwe systemen te ontwerpen en fabriceren, is er een wiskundig model gemaakt van het foton rendement van frequentiedomein gebaseerde FLIM (hoofdstuk 5). Het vermogen van de lichtbron die nodig is voor de belichting in een FLIM systeem en de signaal/ruisverhouding (SNR) van de detector resulteert in een fotonenbudget. Bijvoorbeeld, bij gebruik van fluoresceïne is een lichtbron van slechts een paar milliwatt voldoende voor het FLIM systeem. Per 100 uitgezonden fotonen wordt slechts één foton omgezet in een foto-elektron, hetgeen ons brengt tot een schatting van de ideale SNR van 5x (14dB)

voor één fluorescenemolecuul in de afbeelding. We hebben experimenten uitgevoerd om de parameters en veronderstellingen van het wiskundige model te valideren. De transmissie-efficiëntie in de optische keten van lenzen, filters en spiegels kunnen worden beschouwd als parameters met constante waarden. De wet van Beer-Lambert is van toepassing voor het bepalen van de absorptie factor in het wiskundige model. De aanname van de Poisson-verdeling, die gebruikt is bij het afleiden van de SNR, is ook geldig.

We hebben compacte FLIM systemen gebouwd op basis van nieuwe ontwerpen CCD-beeldsensors met mogelijkheid van demoduleren op pixelniveau. Twee verschillende modellen worden geïntroduceerd (hoofdstuk 6): De horizontaal schakelende MEM-FLIM1 camera en de verticaal schakelende MEM-FLIM2 camera. Met de camera evaluatietechnieken zoals beschreven in hoofdstuk 7 zijn deze twee versies van de MEM-FLIM systemen uitgebreid onderzocht en vergeleken met het FLIM systeem gebaseerd op de conventionele beeldversterker (hoofdstuk 8). De lage verticale ladingstransportefficiëntie beperkt de MEM-FLIM1 camera om lifetime metingen uit te voeren, maar de MEM-FLIM2 camera blijkt een succes. De MEM-FLIM2 camera geeft niet alleen vergelijkbare lifetime resultaten in vergelijking met de “beeldversterker gebaseerde” referentie camera, maar toont ook een veel betere beeldkwaliteit en laat meer gedetailleerde structuren zien in de biologische preparaten. De nieuwe MEM-FLIM systemen kunnen de acquisitietijden verkorten omdat twee fasen gelijktijdig opgenomen worden.

De MEM-FLIM2 camera is echter niet perfect; hij kan alleen worden gemoduleerd op een enkele frequentie (25 MHz) en vereist dat de lichtbron tijdens uitlezing uitgeschakeld wordt. Dit is het gevolg van een onbedoelde kleine afwijking van een aluminiummasker op de CCD-chip. Een herontwerp van de architectuur gebaseerd op het verticaal schakelende concept bracht ons tot de MEM-FLIM3 camera (hoofdstuk 9). Meerdere verbeteringen zijn doorgevoerd in het ontwerp van de CCD-sensor voor de MEM-FLIM3 camera, zoals hogere vulfactor, groter aantal pixels, enz. De MEM-FLIM3 camera kan hogere frequenties aan (40, 60 en 80 MHz) en heeft een optie voor elektronenmultiplicatie. Evaluaties van dit bijgewerkte MEM-FLIM systeem worden gepresenteerd (hoofdstuk 10). De verkregen afbeeldingen met de MEM-FLIM3 camera op 20 en 40 MHz kunnen direct gebruikt worden voor de lifetime berekeningen en de verkregen lifetime resultaten zijn vergelijkbaar met die van de referentie-camera. Er zijn echter verschillen in de even en oneven kolommen (20 MHz) en vier beeldsegmenten (40 MHz) voor zowel de intensity en lifetime opnamen. Bij gebruik van hogere frequenties (60 en 80 MHz) zijn calibraties nodig die vooraf gaan aan de berekening van de lifetimes. De lifetime, die is afgeleid vanuit de modulatie diepte, is na kalibratie redelijk binnen bereik terwijl de lifetime die is afgeleid vanuit de fase onbruikbaar is. Op 60 en 80 MHz kunnen we voor de levensduurberekening gebruik maken van één faseregister van de MEM-FLIM3 camera, op dezelfde manier zoals de referentie-camera werkt. De volgens deze methode verkregen lifetimes uit de MEM-FLIM3 camera, op zowel 60 en 80 MHz, zijn vergelijkbaar met die van de referentie-camera. De MEM-FLIM3 camera heeft ook een elektron-multiply functie voor lowlight experimentele condities. De verkregen multiplicatie was ongeveer 500 maal. Door de beperkte tijd in het project is de lifetime meting met EM-functie niet getest.

Biography

Qiaole Zhao was born on Jan 15, 1984 in Taiyuan, China.

She received her Bachelor's degree in Electronic Science and Engineering from Southeast University, Nanjing, China, in 2006.

In 2006, she started her Master study in the Micro-Electronic-Mechanical System (MEMS) lab in Southeast University. In 2007, she worked at the MEMS lab in the National Cheng Kung University in Taiwan for four months as an exchange student. In 2009, she got her Master degree in Electronic Science and Engineering in Southeast University in Nanjing.

From Feb 2009, she started her Ph.D. project at the Quantitative Imaging Group in Department of Imaging Science and Technology (Department of Imaging Physics), Faculty of Applied Sciences at the Delft University of Technology, in the Netherlands. She was supervised by Prof. dr. I.T. Young and worked in close collaboration with Teledyne Dalsa in Eindhoven, Lambert Instruments in Roden, and Netherlands Cancer Institute in Amsterdam. Her Ph.D. research was about evaluating a new FLIM (Fluorescence Lifetime Imaging Microscopy) system based upon a pixel level modulated camera. This work was funded by Innovation-Oriented Research Program (IOP) of The Netherlands (IPD083412A).

Qiaole has continued her career as a Processing Geophysicist in Shell Global Solutions International B.V. (Rijswijk, the Netherlands) from Jan 2014.

List of publications

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