Transient State Monitoring and Fluorescence Correlation Spectroscopy of Flavin Adenine Dinucleotide

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Master of Science Thesis in Medical Engineering
Stockholm 2014
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TRAST och FCS mikroskopi av flavin-adenin-dinukleotid

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Master of Science Thesis in Medical Engineering
Advanced level (second cycle), 30 credits
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TRITA-STH. EX 2014:82

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Abstract

Many human diseases including cancer have been associated with altered cellular metabolism and a changed oxygen consumption in cells. Fluorophores are sensitive to their local environment due to their long life times in transient dark states. A recent study successfully utilized this sensitivity to image differences in oxygen concentrations in cells using transient state (TRAST) microscopy together with fluorescent labels [1]. A natural continuation of this study is to investigate the possibilities of using this method with natural fluorophores already present in cells and thereby avoid artificial labeling.

Flavin adenine dinucleotide (FAD) is an autofluorescent coenzyme that is naturally present in cells and involved in cellular metabolism. This project is an exploratory pilot study for cellular measurements with the aim to investigate if FAD can be used to probe oxygen concentrations in aqueous solution using transient state monitoring and fluorescence correlation spectroscopy (FCS). This thesis includes the results from FCS and TRAST experiments on FAD in aqueous solutions with different oxygen concentrations as well as different ascorbic acid concentrations. The performed experiments showed that FAD monitored with TRAST is sensitive to differences in oxygen concentrations for the aqueous solutions used in this study.
Sammanfattning

Många sjukdomar hos människor såsom cancer har sammankopplats med förändrad cellmetabolism och en förändring i cellernas syrekonsumtion. Fluoroforer besitser en känslighet mot sin omgivande miljö tack vare sina långa livstider i transienta mörka tillstånd. Denna känslighet har framgångsrikt använts med hjälp av inmärkta fluoroforer i en nyligen publicerad studie där TRAST (transient state) mikroskopi användes för att mäta skillnader i syrekoncentration hos celler [1]. En naturlig uppföljning av denna studie är att undersöka möjligheterna att använda denna metod med fluoroforer som finns naturligt i celler och därigenom undvika tillsatta fluoroforer.

Flavin-adenin-dinukleotid (FAD) är ett autofluorescent koenzym som förekommer naturligt i celler och medverkar i cellens metabolism. Detta arbete utgör en förstudie inför cellulära mätningar med huvudmålet att undersöka huruvida FAD kan användas för att mäta syrekoncentrationer i vattenlösningar med TRAST och FCS (fluorescence correlation spectroscopy). I rapporten redovisas resultat från FCS- och TRAST-mätningar på FAD i vattenlösningar med olika syrekoncentrationer, samt med olika koncentrationer av ascorbinsyra. De utförda experimenten visar att det genom TRAST-mätningar på FAD är möjligt att urskilja skillnader i syrekoncentrationer mellan de vattenlösningar som används i denna studie.
Chapter 1

Introduction

Altered metabolism in cells has recently gained new focus and has been accepted among researchers as a hallmark of cancer [2, 3]. Altered cellular metabolic function has also been associated with many other human diseases including diabetes, obesity and neurodegeneration [3]. In cancer cells the cellular oxygen consumption is often affected and cancer cells tend to use less oxygen compared to healthy cells in relation to their energetic needs [1, 2]. This is commonly referred to as the Warburg effect. The metabolic state of cells is an important health indicator and the development of efficient methods to image metabolic activity could result in a better understanding on all biological levels, as well as powerful diagnostic tools for detection of early staged diseases.

Fluorophores show a sensitivity to their micro-environment due to the long life times in transient dark states, that last long enough to allow interaction with surrounding molecules. It is therefore interesting to study if fluorophores can be utilized as sensors in order to monitor local micro-environments that can provide important information about cells metabolism and overall health.

The environment sensitivity of fluorophores has been successfully utilized by Spielmann et al., who have monitored differences in oxygen concentrations in cells using transient state microscopy (TRAST) with fluorescent labels [1]. The technique exploits the sensitivity of the life time in transient dark states, in particular the triplet state, in order to probe oxygen concentrations in cells and opens up new possibilities in cancer diagnosis. However, labeling with artificial fluorophores can be time consuming and adds complexity to sample preparations. It remains to investigate the possibilities of using transient state monitoring with natural fluorophores, which would circumvent the need of labeling.

This project is an exploratory pilot study for such cellular measurements on the autofluorescent flavin FAD (flavin adenine dinucleotide), which is naturally present in cells and involved in cellular metabolism. It is investigated
if FAD can be used as a sensor to monitor differences in oxygen concentrations in aqueous solution using transient state monitoring and fluorescence correlation spectroscopy (FCS).

This study constitutes a small part of a larger experimental project with the main goal to investigate the possibilities of using FCS and TRAST on FAD in order to probe oxygen consumption in cells without labeling. The next step in the larger project will be cellular measurements and if this technique shows to be successful in cells, it could become an invaluable tool in medicine to diagnose cancer and perhaps also other diseases related to metabolic disturbances in cells.

As this study is a part of a project that attempts to evaluate if this technique can be used as a sensor of oxygen concentrations in living cells, the primary goal of this study is neither to measure absolute values of parameters describing the photophysics of FAD, nor to try to explain in detail the underlying photophysics behind the observations, but rather to investigate if it is possible to resolve differences in oxygen concentrations between different environments with TRAST and FCS.
Chapter 2

Theoretical Background

In this chapter a brief description of flavin adenine dinucleotide and its relevant photophysical properties is given and complemented by a short introduction to the basic principles of fluorescence correlation spectroscopy and transient state monitoring.

2.1 Flavin Adenine Dinucleotide

FAD is a coenzyme of riboflavin and is important in several metabolic processes in cells. It is autofluorescent and consists of an isoalloxazine ring connected to a ribityl adenine diphosphate [4]. By alternating between its oxidized state, FAD, and its reduced state, FADH$_2$, the flavin serves as an electron carrier in the ATP production in the mitochondria [5].

2.1.1 Chemical and Photophysical Properties

The isoalloxazine ring is responsible for the absorption and fluorescence to and from the first excited singlet state and determines the shape of the fluorescent spectrum of FAD, as well as of the chemically closely related riboflavin and flavin mononucleotide (FMN), which show a similar shape in their fluorescence spectra [6]. For the structural formulae of FAD, FMN and riboflavin see Figure 2.1.

FAD is fluorescent in its oxidized form, but hardly fluorescent in its reduced forms [4,6,7]. FAD has a typical lifetime of 2.7 ns and a quantum yield of 0.03, which is about ten times lower than the quantum yield of FMN and riboflavin [8–10]. Absorption occurs in the visible region with peaks at 375 nm and 450 nm. The emission peak is broad with a maximum at 525 nm [7,10]. The lower quantum yield of FAD is due its adenine moiety, which forms an intra-molecular complex with the isoalloxazine ring [4,6,9,11]. In this stacked conformation the molecule can undergo efficient, non-radiative decay by electron transfer to the ground state [4,6,9]. In the range from...
CHAPTER 2. THEORETICAL BACKGROUND

Figure 2.1. Chemical structure of flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN) and Riboflavin.

pH 4 to pH 9, about 80\% of the FAD molecules are in this non-fluorescent stacked conformation [6,9,11].

Reduction of Flavin Adenine Dinucleotide

FADH and FADH$_2$ are the reduced forms of FAD and are hardly fluorescent [4]. FAD can reversibly be reduced to FADH$_2$ either by accepting one electron twice in a two step reaction or by accepting two electrons at once. The net reduction reaction is given by:

$$\text{FAD} + 2\text{H}^+ + 2\text{e}^- \rightarrow \text{FADH}_2$$  \hspace{1cm} (2.1)

It is well established that photo-excitation of flavins leads to population of the triplet state through intersystem crossing, and that reduction takes place from the triplet state [12].
2.2 Fluorescence Correlation Spectroscopy

Fluorescence correlation spectroscopy (FCS) was developed in the early 1970s as a method to study molecular relaxation processes [13]. The stochastic fluctuations of the emitted fluorescence from fluorophores, contained in an open volume, are used to extract physical parameters by means of mathematical methods [13]. FCS provides close-to individual molecule resolution and yields valuable information such as the number of molecules contained in the observation volume, speed of diffusion and photophysical parameters [11].

To be able to distinguish the small fluctuations, the technique requires highly sensitive detectors with high temporal resolution. It is important that the fluorophore concentration is sufficiently small (typically in the nanomolar range) and the detection volume is small, so that the volume contains only very few fluorophores, as a large ensemble yields a constant signal and the statistical information is lost.

2.2.1 Correlation Analysis

In an FCS experiment, the raw data is called the time trace and consists of the number of photons detected at each time \( t \). The detected fluorescence intensity, \( F(t) \), origins from excited molecules inside the observation volume and is proportional to the number of fluorophores contained in the volume at time \( t \). The detection volume is illustrated in Figure 2.2.

In a simplified system with no bleaching or dark states, each fluorophore within the observation volume contributes to the fluorescence until it diffuses
out of the detection region. The diffusion of fluorophores in and out of the volume will cause fluctuations in the detected intensity. In the presence of a dark transient state, for example a triplet state, additional fluctuations will be generated as the fluorescence will alternate between "on" and "off" as the molecules traverse the volume. This is visualized in Figure 2.3.

To analyze the data, a theoretical model of the fluctuations is needed. From the time trace of the detected fluorescence, a correlation function, depending on the time difference, $\tau$, between two intensity recordings at $t = 0$ and $t = \tau$, can be calculated:

$$G(\tau) = \frac{\langle F(t)F(t+\tau) \rangle}{\langle F(t) \rangle \langle F(t) \rangle} = \frac{\langle \delta F(0)\delta F(\tau) \rangle}{\langle F(\tau) \rangle ^2} + 1 \quad (2.2)$$

The brightness, $B$, is a measure of the signal that can be expected from emitted fluorescence of a single fluorophore and is given by:

$$B = q\sigma Q \quad (2.3)$$

where $\sigma$ is the absorption cross-section, $Q$ the fluorescence quantum yield and $q$ the quantum efficiency of detection of emitted photons, which depends on the experimental conditions, including light intensity, efficiency of the light collecting system and the detectors. The detected intensity depends on the spatial distribution of the excitation laser and detection efficiency in the volume. This is accounted for by the collection efficiency function $CEF(\vec{r})$ [14], and the detected fluorescence is given by integration over the observation volume:
2.2. FLUORESCENCE CORRELATION SPECTROCOPY

\[ F(t) = B \int_V \text{CEF}(\vec{r}) I(\vec{r}) C(\vec{r}, t) \, dV \]  

(2.4)

Here, \( C(\vec{r}, t) \) is the spatial distribution of fluorophores and \( I(r) \) is the excitation intensity at position \( \vec{r} \). With a Gaussian beam the illuminated spot is well approximated by a three-dimensional Gaussian distribution given by

\[ I(x, y, z) = I_0 \exp \left( -2 \frac{x^2 + y^2}{\omega_{xy}^2} \right) \exp \left( -2 \frac{z^2}{\omega_z^2} \right) \]  

(2.5)

where the \( z \)-axis coincides with the optical axis of the laser beam [14]. The radii, \( \omega_{xy} \) and \( \omega_z \), in the \( xy \) - and \( z \)-direction, respectively, are taken at the surface where the excitation intensity profile reduces to \( e^{-2} \) of its maximal value \( I_0 \).

In a sample where fluctuations origin from free translational diffusion only, the autocorrelation function is given by

\[ G(\tau) = \frac{1}{N} \left( 1 + \frac{4D\tau}{\omega_{xy}^2} \right)^{-1} \left( 1 + \frac{4D\tau}{\omega_z^2} \right)^{-1/2} + 1 \]  

(2.6)

where \( N \) is the number of molecules and \( D \) the diffusion coefficient of the species. The average time for a molecule to stay inside the observation volume before it exits by diffusion is called diffusion time, \( \tau_D \), and is related to the diffusion coefficient by:

\[ \tau_D = \frac{\omega_{xy}^2}{4D} \]  

(2.7)

Using this relation, Equation (2.6) can be rewritten as

\[ G(\tau) = \frac{1}{N} \left( 1 + \frac{\tau}{\tau_D} \right)^{-1} \left( 1 + \frac{\tau}{\tau_D s^2} \right)^{-1/2} + 1 = \frac{1}{N} G_D(\tau) + 1 \]  

(2.8)

where \( s = \omega_z/\omega_{xy} \) is called the structure parameter and \( G_D \) refer to the part of the autocorrelation function that relates to the diffusion time. Other sources of intensity fluctuations may come from various types of processes, and require different models. In the case of a present triplet state, with intersystem crossing from the the first excited state to the lowest triplet state, the autocorrelation function becomes

\[ G(\tau) = \frac{1}{N(1 - \bar{T})} \left[ 1 - \bar{T} + \bar{T} \exp \left( \frac{-\tau}{\tau_T} \right) \right] G_D(\tau) + 1 \]  

(2.9)

where \( \bar{T} \) denotes the average fraction of the observed molecules that populates the triplet state and \( \tau_T \) is the triplet state relaxation time.
2.3 Transient State Monitoring

Transient state monitoring is a relatively new technique introduced by Sandén, Persson and Widengren [15, 16] and is based on a mathematical model of fluorophore relaxation from excited states back to their ground state upon pulsed light excitation. By measuring the time averaged response to excitation laser pulses of different lengths, kinetic parameters of the transient states, such as the triplet decay rate, can be calculated [17]. The requirement of temporal resolution in FCS is transferred from detection to excitation in TRAST, through modulation of the excitation laser, which enables TRAST to be used with charge-coupled device (CCD) cameras that can provide spatial information.

2.3.1 The Three-State Electronic Model

Given a three-state electronic model, as presented in the Jablonski energy diagram in Figure 2.4, the population dynamics of the singlet ground state, $S_0$, the first singlet excited state, $S_1$, and the lowest triplet state, $T_1$, at position $\vec{r}$ and time $t$, under continuous constant excitation, can be described by a system of ordinary differential equations, here written in matrix form:

$$\frac{d\vec{X}}{dt} = \mathbf{A}\vec{X}$$

where $\vec{X}$ denotes a vector consisting of the three states. The excitation rate, $k_{01}$, is dependent on the excitation intensity, $I(\vec{r}, t)$, as well as the excitation intensity, $I(\vec{r}, t)$, as well as the excitation...
2.3. TRANSIENT STATE MONITORING

cross-section, $\sigma_{\text{exc}}$, for transitions from $S_0$ to $S_1$:

$$k_{01}(\vec{r}, t) = \sigma_{\text{exc}} I(\vec{r}, t) \quad (2.11)$$

Taking $S_0$, $S_1$ and $T_1$ to be probabilities that sum to one:

$$[S_0(\vec{r}, t) + S_1(\vec{r}, t) + T_1(\vec{r}, t)] = 1 \quad (2.12)$$

the initial condition before laser excitation, assuming that all fluorophores are in their ground state, becomes

$$\begin{bmatrix} S_0(\vec{r}, t_0) \\ S_1(\vec{r}, t_0) \\ T_1(\vec{r}, t_0) \end{bmatrix} = \begin{bmatrix} 1 \\ 0 \\ 0 \end{bmatrix} \quad (2.13)$$

at $t_0 = 0$. This condition also applies to the situation when the fluorophore have not been exposed to excitation for a sufficiently long time before $t_0$, such that all fluorophores have been given enough time to fully relax back to $S_0$ from higher energy states. Solving Equation (2.10) yields the population in the three states. The time evolution of the populations in the singlet excited state and the lowest triplet state is given by:

$$S_1 = \frac{k_{01} k_T}{k_{01}(k_{\text{ISC}} + k_T) + k_{10} k_T} e^{\lambda_1(\vec{r}) t} - \frac{k_{01}}{k_{01} + k_{10}} \exp^{\lambda_2(\vec{r}) t}$$

$$+ \frac{k_{01}^2 k_{\text{ISC}}}{(k_{01} + k_{10}) [k_{01}(k_{\text{ISC}} + k_T) + k_{10} k_T]} \exp^{\lambda_3(\vec{r}) t} \quad (2.14)$$

$$T_1 = \frac{k_{01} k_{\text{ISC}}}{k_{01}(k_{\text{ISC}} + k_T) + k_{10} k_T} e^{\lambda_1(\vec{r}) t} - \frac{k_{01} k_{\text{ISC}}}{k_{01}(k_{\text{ISC}} + k_T) + k_{10} k_T} \exp^{\lambda_2(\vec{r}) t} \quad (2.15)$$

where $\lambda_1$, $\lambda_2$ and $\lambda_3$ are the eigenvalues of matrix $A$, given by:

$$\lambda_1 = 0 \quad (2.16)$$

$$\lambda_2 = -\frac{1}{2}(k_{01} + k_{10} + k_{\text{ISC}} + k_T) + \frac{1}{2}[(k_{01} + k_{10} + k_{\text{ISC}} + k_T)^2$$

$$- 4(k_{01} k_{\text{ISC}} + k_{01} k_T + k_{10} k_T + k_{\text{ISC}} k_T)]^{1/2} \quad (2.17)$$

$$\lambda_3 = -\frac{1}{2}(k_{01} + k_{10} + k_{\text{ISC}} + k_T) - \frac{1}{2}[(k_{01} + k_{10} + k_{\text{ISC}} + k_T)^2$$

$$- 4(k_{01} k_{\text{ISC}} + k_{01} k_T + k_{10} k_T + k_{\text{ISC}} k_T)]^{1/2} \quad (2.18)$$

Since $\lambda_1$ is zero and the remaining eigenvalues are negative, the first term of $S_1$ is time independent and a steady state will be reached as $t \to \infty$, which means that the total population is conserved when no bleaching occurs. The
second eigenvalue, $\lambda_2$, is related to the antibunching relaxation time, $\tau_{\text{AB}}$, which is the relaxation time for equilibration of the populations between the two singlet states. The third eigenvalue, $\lambda_3$, is related to the build-up time for the triplet state and the triplet relaxation time, $\tau_T$, is given by its inverse:

$$\tau_T = -1/\lambda_3 \quad (2.19)$$

In TRAST the excitation intensity, $I(\vec{r})$, is modulated, as illustrated in Figure 2.5, to give a square-wave pulse train with $n$ pulses of length $t_p$ and a period time $T$:

$$I(\vec{r}, t) = \begin{cases} I(\vec{r}), & \text{if } 0 < t \leq t_p \\ 0, & \text{if } t_p < t \leq T \end{cases} \quad (2.20)$$

The time-averaged fluorescence from the sample reflects the population of $S_1$ through the relation

$$F = \Phi_f k_{10} S_1(\vec{r}, t) \quad (2.21)$$

where $\Phi_f$ is the fluorescence quantum yield. With an alternating excitation intensity and a confocal detection identical to that used in FCS, the average fluorescence from the observation volume during a pulse train can therefore be described by:

$$\langle F \rangle = \frac{1}{n \cdot T} \int_V \text{CEF}(\vec{r}) c k_{10} \Phi_f \Phi_D \sum_{i=1}^n \left[ \int_0^{t_p} S_{1,i}^\text{on}(\vec{r}, t) \, dt + \int_{t_p}^{T} S_{1,i}^\text{off}(\vec{r}, t) \, dt \right] \, dV \quad (2.22)$$

Here, $\Phi_D$ is the detection quantum yield of the instrument, CEF is the collection efficiency function and $c$ is the concentration of fluorescent molecules. The population probabilities $S_{1,i}^\text{on}(\vec{r}, t)$ and $S_{1,i}^\text{off}(\vec{r}, t)$ describe the probability that a fluorophore is in its first excited singlet state at time $t$ and position $\vec{r}$ during the pulse ($0 < t < t_p$) and between pulses ($t_p < t < T$), respectively. Their full expressions are omitted in this thesis and the interested reader
is referred to the proof-of-principle published by Sandén et al., 2007 [15], which this chapter is largely based on.
Chapter 3

Methods and Materials

This chapter covers the methods and materials used to perform the experiments including a description of how the samples were prepared, a detailed description of the setup, and a description of the experimental methods and data analysis for FCS and TRAST experiments, respectively.

3.1 Sample Preparations

Experiments were performed on FAD in aqueous solution with different oxygen concentrations. Three different conditions were tested: oxygen-saturated samples, air-saturated samples, and oxygen-free samples.

The flavin FAD (flavin adenine dinucleotide, disodium salt hydrate – ≥95% HPLC, powder #F6625) was purchased from Sigma-Aldrich. Potassium phosphate buffer 50 mM, with a pH of 7.2, was used in all experiments. Fresh samples were prepared each day from frozen stock solutions of 100 µM FAD in buffer, stored at −77°C.

The laser was focused through a cover glass into a hanging droplet of 70 µl to 100 µl sample. For measurements exceeding 5 min, the sample was placed under a coverglass in an airtight chamber together with a small water reservoir to minimize effects of evaporation on the sample concentration.

For anaerobic experiments, oxygen was evacuated from the sample by flushing the chamber with argon, led through a bubble humidifier, for at least 30 min before the measurements were performed. The same procedure was carried out for the fully oxygenated experiments, saturating the sample with oxygen by flushing the chamber with oxygen instead of argon.

3.2 Instrumental Setup

A new confocal setup was established that allowed for both FCS and TRAST measurements. The setup was essentially identical to a standard FCS instrument apart from the addition of an acousto-optic modulator (AOM),
3.2. INSTRUMENTAL SETUP

Figure 3.1. The experimental setup. A typical FCS setup essentially consists of a laser, beam expander, dichroic mirror, emission filter, objective (not included in the figure), detector, correlator and a computer. In addition to these standard components the beam was led through a laser power controller (LPC) followed by an acousto-optic modulator (AOM), required for the TRAST experiments, which was inactivated for FCS applications.

A krypton-argon laser (Omnichrome series, 43 643-RYB-A02) was used together with a narrow 488 nm clean-up filter (full-width half maximum 1.9 nm, Semrock). The power was controlled with a laser power controller (Melles Griot, LPC-VIS) before the beam was focused by a convex lens ($f=175$ mm) into the AOM (MT200-A0.5-VIS, AA Opto-electronic). The AOM was computer controlled to produce a pulsed, square-wave excitation when the setup was used for TRAST measurements, and is described in more detail in Section 3.2.1. Another convex lens ($f=500$ mm) was placed after the AOM to recollimate the beam and act as a beam expander together with the first lens. The magnified beam was then focused by a lens into an epi-illuminated microscope (Leitz-Wetzlar), reflected by a dichroic mirror (FF495-Di03, Semrock) and focused into the sample by a water immersion objective (Zeiss 461832 Plan-Neofluar 63× NA 1.2).

The redshifted fluorescence from the sample was collected back into the objective and led through a 50 μm pinhole and divided by a beam splitter, directing the light towards two avalanche photodiode (APD) detectors (PerkinElmer Optoelectronics, SPCM-AQR-14). The pinhole, which was positioned in the image plane, minimizes the observation volume by rejecting out-of-focus light, especially in the axial direction.

Preceding the two detectors, two band-pass emission filters (530/55, Semrock) were inserted to eliminate background light, especially leaking...
excitation laser and Raman scattering from water molecules in the sample.

The beam waist, $\omega_{xy}$, was determined to 0.33 $\mu$m. This was obtained from FCS measurements on the standard reference fluorophore rhodamine 110 in water, by measuring the diffusion time and applying Equation (2.7) with the insertion of the diffusion coefficient, $D = (4.7 \pm 0.4) \times 10^{-6} \text{cm}^{-2} \text{s}^{-1}$, taken from Reference [18].
3.2. INSTRUMENTAL SETUP

3.2.1 Amplitude Modulation

An AOM can be used to modulate the beam and produce a pulsed, square-wave beam. The AOM is essentially a crystal with an attached piezoelectric transducer that generates acoustic waves when an RF-signal is applied. The acoustic waves traveling through the crystal act like a phase grating, diffracting the incident laser beam into a number of interference maxima. At a certain angle of incidence, the AOM will operate in a so-called Bragg mode configuration and produce only the first order diffraction beam, since the other cancel out by destructive interference [19], see Figure 3.2.

The zeroth- and first-order beams are separated by the wavelength-dependent separation angle, \( \Delta \theta = \frac{\lambda f}{v} \), where \( f \) is the acoustic frequency, \( v \) the acoustic velocity in the crystal and \( \lambda \) the wavelength of the laser beam [19]. For this setup the separation angle was calculated to be \( 23.2 \text{ mrad} \).

When the AOM is activated, a large part of the beam is diffracted to the first order and thereby diverges with an angle of \( \Delta \theta \) from the path of incidence. When the AOM is inactivated, the beam will pass through the AOM undiffracted. By selecting only the first order beam to pass through a subsequent aperture, the AOM can be controlled by a computer to modulate the beam and produce square-waved pulse trains.

The rise time, \( T_r \), of the AOM, is defined as the time it takes for the beam intensity to increase from 10% to 90% of the maximum intensity, and is related to the time required for the sound wave to traverse the beam, and hence, it is related to the acoustic velocity in the crystal and the beam diameter, \( \varphi \), through the equation:

\[
T_r = 0.66 \frac{\varphi}{v} \quad (3.1)
\]

To optimize the rise time, the beam was focused into the AOM by the two lenses. To avoid damage of the AOM, considerations was taken to the maximum optical power density of the AOM, which sets an upper limit to the lens strength that can be used. For this setup the rise time was measured with an oscilloscope to be \( \leq 40 \text{ ns} \). An AOM is not required for FCS appli-
cations, and is normally not included in an FCS setup, but since it—for the purpose of this study—was desirable to let the beam path be identical for both TRAST and FCS measurements, the laser beam was guided through the AOM. For the FCS experiments the AOM was set to a continuous mode, diffracting the beam without modulating the beam intensity.

3.3 Experiments and Data Analysis

3.3.1 Fluorescence Correlation Spectroscopy Experiments

Three series, each comprising five to six FCS experiments, were carried out using excitation laser intensities between 15 kW cm\(^{-2}\) and 240 kW cm\(^{-2}\) for oxygen-saturated, air-saturated and oxygen-free buffer solutions containing 500 nM FAD. Due to the low count rate observed from FAD measurements (≈ 18 counts/s observed by each of the two detectors at the lowest excitation intensity used), the FCS experiments required long measuring times, up to one hour for the lowest excitation intensity in air. A shorter acquisition time could be used for measurements with higher laser excitation intensities, and at the highest intensity used, the acquisition time could be decreased to twenty minutes.

Additionally, a set of FCS experiments were performed with different concentrations of the reducing agent ascorbic acid, varying the concentration between 0 mM and 1 mM, at an excitation intensity of 92 kW cm\(^{-2}\). The FAD concentration was 500 nM in all experiments. Sample preparations were performed directly before each measurement in order to avoid sample degradation as much as possible. The data was correlated by a PC-based ALV-5000/E correlator in fast correlation mode.

The data from the FCS experiments was fitted and analyzed in Matlab, using a trust region, nonlinear least squares algorithm to produce the fits. In the fitting procedure, the model described in Equation (2.9), which includes both diffusion and one triplet state, was used. Generous boundaries were given to the involved parameters \(N, T, s, \tau_D\) and \(\tau_T\) so that they could vary close to freely. For the experiment performed on the oxygen-saturated sample at the lowest laser excitation intensity, the triplet population was too low to be accurately determined, and a model that only includes the process of diffusion, given in Equation (2.8), was used to fit the data. Only data points with \(\tau\) in the range \(5 \times 10^{-8} \text{s} \leq \tau \leq 1 \text{s}\) were used in the fitting procedure.

3.3.2 Transient State Monitoring Experiments

Power series of four to five TRAST experiments on 4 \(\mu\)M FAD in oxygen-saturated, air-saturated and oxygen-free buffer solutions were performed with excitation laser intensities within a range of 18 kW cm\(^{-2}\) to 92 kW cm\(^{-2}\).
3.3. EXPERIMENTS AND DATA ANALYSIS

Transient State Monitoring

- Modulated excitation
- Monitor dark state build-up by observing time averaged fluorescence

\[ F \propto \frac{k_{2175}}{g_{2778}} \]

Figure 3.3. The data collected from the TRAST experiments were fitted using a model that included a reduced state \((R)\), which is formed from the lowest triplet state.

Since the FAD concentration was held constant throughout all the experiments, the upper limit of the excitation intensity was determined by the detectors, which have an upper limit to their linear region of photon detection.

A series of experiments with concentration of ascorbic acid ranging from 0 mM to 1 mM was also carried out. The samples were prepared immediately before each experiment to avoid sample degradation.

For each experiment, the samples were exposed to a set of pulse trains with different pulse lengths and the detectors recorded during the full experiments. In each set of pulse trains, the pulse length was stepped between 0.1 µs and 1 ms, with a duty cycle of 1% (the percentage of the pulse train in which the excitation laser is on, \(\eta = t_p/T\)). A pause of 1 s was added between each pulse train. The total illumination time was kept constant for all pulse trains in each experiment, but was varied between experiments with different excitation intensities. For experiments with low excitation intensities of 18 kW cm\(^{-2}\) and 37 kW cm\(^{-2}\) an illumination time of 300 ms and 200 ms was used, respectively. A shorter illumination time of 100 ms was used for the remaining experiments. The AOM, which produced the pulse trains, was computer controlled through a Matlab script.

The collected data from the TRAST experiments was analyzed with a Matlab program, which simulated both the excitation profile and the detected fluorescence in order to calculate the TRAST curves.

A nonlinear least squares method was used to fit the model to the observed data, letting the rate constants vary freely. The model contained one triplet state and one dark reduced state, \(R\), with the reduction occurring from the triplet state, as shown in Figure 3.3. The reduced state introduces two new rate constants: the rate of the transition from the triplet state to the reduced state, \(k_{off}\), and the rate of the transition from the reduced state back to the ground state, \(k_{on}\).
Chapter 4

Results

In this chapter the results from the performed experiments are presented. FCS experiments are presented in Section 4.1 and TRAST experiments are presented in Section 4.2. For the sake of clarity, some of the experimental data are omitted in this chapter and can be found in Appendix A, which contains the collected data and fitted curves for the complete sets of experimental data. Both the FCS and TRAST measurements showed that the dynamics of the triplet state of FAD is sensitive to oxygen concentration and differences in oxygen concentration could be resolved.

4.1 Fluorescence Correlation Spectroscopy Experiments

The correlated data from FCS experiments of 500 nM FAD in oxygen-saturated, air-saturated and oxygen-free 50 mM potassium phosphate buffer solutions, under a laser excitation intensity of 92 kW cm$^{-2}$, is shown in Figure 4.1 together with the fitted curves. The fit was done using a correlation model, with diffusion and one triplet state, given in Equation (2.9). The aerobic experiments show two distinct decays in their autocorrelation curves, where the first decay is in the microsecond time range and indicates a build-up of a dark transient state. Reviewing the three-state electronic model, as described in Section 2.3.1, this corresponds to a build-up in the triplet state. The second decay is in the millisecond time range and is attributed to the process of diffusion. For the anaerobic environment the curve shows a large dark state build-up and the decay is no longer well separated from the decay of the diffusion, which makes it difficult to determine the involved parameters.

The estimated parameters, the triplet state relaxation time $\tau_T$, the population of the triplet state, $T$, expressed as a fraction, and the counts per molecule (CPM), for power series experiments in the three differently oxygenated environments, are presented in Figure 4.2.
4.1. FCS EXPERIMENTS

Figure 4.1. FCS correlation curves of 500 nM FAD in oxygen-saturated, air-saturated and oxygen-free 50 mM potassium phosphate buffer solutions, at excitation intensity 92 kW cm$^{-2}$. Fits are shown as solid lines.

Figure 4.2. Calculated photophysical parameters from FCS measurements of 500 nM FAD at different excitation intensities in oxygen-saturated, air-saturated and oxygen-free (argon) 50 mM potassium phosphate buffer solutions. (a) The triplet state relaxation time, $\tau_T$. (b) The triplet state population, $T$. (c) Counts per molecule, (CPM)
Figure 4.3. FCS correlation curves of 500 nM FAD in 50 mM potassium phosphate buffer solutions, with different concentrations of ascorbic acid (0 mM to 1 mM) at excitation intensity 92 kW cm$^{-2}$. The fits are shown as solid lines.

The triplet relaxation time (Figure 4.2a) shows no dependence on power but shows a large sensitivity to the oxygen concentration of the environment. At 92 kW cm$^{-2}$, the triplet relaxation time is determined to $(8.47 \pm 0.75)$ µs in anaerobic environment, about six times longer than in air, which was determined to $(1.39 \pm 0.26)$ µs. In the oxygen-saturated sample the relaxation time is even shorter, $(0.45 \pm 0.07)$ µs. It is apparent that the triplet relaxation time decreases in the presence of oxygen.

Figure 4.2b shows a slight increase of the triplet state population with increasing excitation power. For the anaerobic environment the population in the triplet states is $(41.0 \pm 3.5)$ % compared to $(18.1 \pm 1.2)$ % in air- and $(19.5 \pm 1.2)$ % in oxygen-saturated environment.

Experiments on FAD in solutions with different concentrations of ascorbic acid, performed in normal air conditions, are presented in Figure 4.3. The apparent number of molecules in the observation volume decreases with increasing reducing agent concentrations. This result was expected, since the ascorbic acid increases the fraction of reduced FAD, which are hardly fluorescent. The correlation curve obtained with low concentration of ascorbic acid, 0.3 mM, resembles in its shape, the curve obtained without reducing agent. The decay in the microsecond time range, related to the triplet state, is clearly separated from the decay related to the diffusion. At ascorbic acid concentrations higher than 0.4 mM, the decay in the microsecond range is no longer well distinguishable. This is probably due to overshadowing by the slower reduction process initiated by the ascorbic acid.
4.2 Transient State Monitoring Experiments

The normalized fluorescence from TRAST measurements of FAD in oxygen-saturated, air-saturated and oxygen-free solutions with excitation intensity 92 kW cm\(^{-2}\) is shown in Figure 4.4. Measurements of FAD in different concentrations of ascorbic acid are shown in Figure 4.5. The data was fitted with two exponentials, according to the four-state electronic model including a reduced state as described in Section 3.3.2. Here, the first exponential is related to a fast process occurring in the microsecond time range corresponding to the triplet-state dynamics of FAD, and the second exponential is likely related to the process of reduction of FAD to FADH\(_2\). The estimated triplet state population, the triplet relaxation time, and the photophysical rate constants observed by TRAST are shown in Figure 4.6.

Some of the curves contained one or two data points that abruptly dropped to values close to zero, and distinctly deviated from the rest of the TRAST curve. The reason for this behavior was not found, but is likely to arise from a bug in the software that controlled the AOM. Since the artifacts were not reproducible when the same experiment was repeated, they were treated as a random errors that did not affect the rest of the collected data. The deviating data points were simply excluded in the fitting procedure.

The rate of intersystem crossing, \(k_{\text{ISC}}\), shown in Figure 4.6a, was determined to \((21.90 \pm 3.28) \mu s^{-1}\) in oxygen-free solution, \((34.00 \pm 3.54) \mu s^{-1}\)

![Figure 4.4. TRAST curves of 4 \(\mu M\) FAD in 50 mM potassium phosphate buffer solutions at excitation intensity 92 kW cm\(^{-2}\) in oxygen-saturated, air-saturated and oxygen-free (argon) samples. Fits are shown as solid lines.](image-url)
in air-saturated solution and \((37.20 \pm 6.45) \mu s^{-1}\) in oxygen-saturated solution, at an excitation intensity of 92 kW cm\(^{-2}\). No statistically significant difference of this rate was found between the two aerobic measurements. However, the intersystem crossing is significantly slower in the anaerobic environment than in the two aerobic environments.

The rate of transition from the triplet state to the ground state, \(k_T\), shown in Figure 4.6b, does not show a dependence on excitation intensity and is in the order of \(\approx 1 \mu s^{-1}\). The rate in oxygen-free, air-saturated, and oxygen-saturated solution is \((0.64 \pm 0.13) \mu s^{-1}\), \((0.88 \pm 0.12) \mu s^{-1}\) and \((1.64 \pm 0.35) \mu s^{-1}\), respectively, at an excitation intensity of 92 kW cm\(^{-2}\). The rate increases consistently with increasing oxygen concentration.

The rate of reduction, \(k_{\text{off}}\), presented in Figure 4.6c, shows a slight increase with applied laser power and shows a similar behavior for all three experimental environments. It was observed to be between \((3 \text{ ms}^{-1}\) and \(10 \text{ ms}^{-1}\)\) at the observed interval of excitation intensities. The rate of oxidation back to the ground state, \(k_{\text{on}}\), shown in Figure 4.6d, was found to be between \(2.9 \text{ ms}^{-1}\) and \(6.1 \text{ ms}^{-1}\) and did not show any dependence on excitation power.

To compare the results acquired by TRAST to the results acquired by FCS, the triplet relaxation time and triplet state population were calculated. The triplet population was estimated by the first term of Equation (2.15), which describes the triplet state population under continuous laser excitation.

![Figure 4.5](image.png)

*Figure 4.5.* Concentration series of TRAST experiments of 4 \(\mu M\) FAD in 50 mM potassium phosphate buffer solutions at excitation intensity 92 kW cm\(^{-2}\) at different concentration of the reducing agent ascorbic acid. Fits are shown as solid lines.
Figure 4.6. Photophysical parameters calculated from TRAST experiments on 4 µM FAD in oxygen-saturated, air-saturated and oxygen-free 50 mM potassium phosphate buffer solution. (a) The rate of intersystem crossing from $S_1$ to $T_1$, $k_{ISC}$. (b) The rate of relaxation from $T_1$ to $S_0$, $k_T$. (c) The rate of reduction from $T_1$ to $R$, $k_{off}$. (d) The rate of reoxidation from $R$ to $S_0$, $k_{on}$. (e) The triplet state relaxation time, $\tau_T$. (f) The triplet state population, $T$. 
at $t \to \infty$, that is, the triplet state population in the steady state:

$$T = \frac{k_{01}k_{\text{ISC}}}{k_{01}(k_{\text{ISC}} + k_T) + k_{10}k_T}$$  \hspace{1cm} (4.1)

The triplet relaxation time was calculated by insertion of the experimentally determined rate constants into Equation (2.18) to yield $\lambda_3$, and then using the relation $\tau_T = -1/\lambda_3$ given by Equation (2.19). No intervals of confidence have yet been calculated for these numbers, and their reliability remains to be investigated. The triplet state relaxation time and the triplet state population are shown in Figure 4.6e and 4.6f, respectively. The triplet state relaxation time shows a similar trend as seen with FCS; it is longer in the anaerobic environment as compared to the two aerobic samples. The triplet population was found to be lower in the oxygen-saturated sample as compared to the less oxygenated samples.
Chapter 5

Discussion and Conclusion

Fluorescence correlation spectroscopy experiments on FAD show a large build-up of dark transient states in anaerobic environments. This build-up decreases with increasing oxygen concentration. The decay on the 1 µs time scale, most evident in the oxygen- and air-saturated samples, is attributed to the triplet state dynamics. The dynamics of the triplet state is also observed in the anaerobic samples, accompanied by an additional decay, of the same order of magnitude as the diffusion, which indicates a second dark transient state with a longer lifetime than the triplet state. This slower process observed is likely related to the reduction of FAD to a reduced state which, in the presence of oxygen, can oxidize back to its ground state in the dark; but in an anaerobic environment, oxidation with oxygen is no longer possible and the process of reoxidation is slowed down or perhaps completely inhibited. This explanation is supported by a study made by Song et al., who have observed permanent photo-reduction of FAD under anaerobic conditions [12].

The reduced states of FAD have weak fluorescence with wavelengths shorter than the range of the emission filters, and appear as dark states when observed with the setup used in this experiment. The suggestion that the observed second decay in the FCS correlation curves is related to the process of reduction, is supported by noting that a second slow process in the same order of magnitude, similar to that observed in the anaerobic samples, can also be observed in the experiments performed with a reducing agent, which show an increased dark state build-up at higher reducing agent concentrations. The speed of this process was observed to be of the same order of magnitude as the diffusion time, which sets the upper limit to the observable time frame. It is therefore difficult to separate these two processes completely from each other. It is also difficult with FCS, especially in the anaerobic experiments, to separate the build-up of the triplet state from the slower process attributed to the reduction, which makes it difficult to determine the triplet state dynamics accurately. The model that was used
to analyse the data accounts only for the diffusion and one triplet state. Even if the model does not account for all involved processes, it appears to fit the data well by examination of the residuals of the correlation function. A better model including the reduction process could perhaps give better parameter estimations and should be considered for future studies. However, this simple model did show itself successful at resolving relative differences in oxygen concentrations of the three differently oxygenated environments tested in this study.

The transient state monitoring experiments reveal two prominent exponential decays that are well separated, where the faster decay corresponds to the triplet state dynamics, and the slower is assumed to be related to the process of reduction. The impact of reduction is clearly seen in the experiments with different reducing agent concentration, where the shape of the TRAST curve distinctly changes with increasing reducing agent concentration, and the process of the second decay is significantly faster at high concentrations of ascorbic acid. The fact that the two decays are much better separated with this method than with FCS, could mean that TRAST is able to provide better parameter estimations of the triplet state dynamics of FAD. The extracted rates related to the triplet state, $k_{ISC}$ and $k_T$, both show a sensibility of oxygen concentrations and can be used to probe oxygen levels of the differently oxygenated environments tested in this project. The calculated triplet population and triplet relaxation time show results comparable to those from FCS experiments showing similar oxygen dependence although not in complete agreement of the absolute values.

\section*{5.1 FAD as an Oxygen Indicator with Transient State Monitoring}

Despite the incapacity of the method to accurately determine absolute values of the parameters, for the purpose of using FAD as a sensor of differently oxygenated environments, it is the ability to resolve relative differences in oxygen concentrations between the samples that are most important. The work presented in this report shows that measurements by TRAST on FAD can separate samples of different oxygen concentrations, and showed results that were in agreement with those achieved by FCS. Consequently, the method showed to be successful in probing relative oxygen concentrations in aqueous solutions.

This project serves as a pilot study for cellular measurements. To evaluate if this method can be used to probe oxygen levels in living cells, this project will be continued with cellular measurements as a next step. The experiments showed that the method was successful with aqueous solutions, under very controlled laboratory conditions, with oxygen concentration as the only variable. The results are promising for further studies, although one
must bear in mind that cells are complex systems and many complications might appear when measuring on living cells. Fluorophores are sensitive to their local environment and fluorescence from other fluorophores present in the cell can disturb the signal from FAD. The question if FAD can be used to probe differences of oxygen concentrations in cells remains to be answered.
Bibliography


flavoenzymes: photochemical and photophysical aspects.”


Appendix A

The Complete Data Sets

A.1 Fluorescence Correlation Spectroscopy Experiments

![Diagram](image)

**Figure A.1.** Power series of FCS experiments on anaerobic samples with 500 nM FAD in 50 mM potassium phosphate buffer solution. Fits are shown as solid lines.
A.1. FCS EXPERIMENTS

Figure A.2. Power series of FCS experiments on air-saturated samples with 500 nM FAD in 50 mM potassium phosphate buffer solution. Fits are shown as solid lines.

Figure A.3. Power series of FCS experiments on oxygen-saturated samples with 500 nM FAD in 50 mM potassium phosphate buffer solution. Fits are shown as solid lines.
A.2 Transient State Monitoring Experiments

Figure A.4. TRAST curves of 4 µM FAD in 50 mM potassium phosphate buffer solution. Fits are shown as solid lines. (a) Power series of air-saturated sample. (b) Power series of anaerobic sample. (c) Power series of oxygen-saturated sample. (d) Concentration series with varying concentration of the reducing agent ascorbic acid (AA).