

Nonlinear laser microscopy to visualize cancer *in vivo*

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Abstract

This report discusses the research of my bachelor thesis in the Molecular Biophysics Group of the Utrecht University. They are building a setup to image cancer *in vivo* with a high power pulsed laser. The laser induces two-photon excitation and second-harmonic generation in the sample. The light that is created by these two nonlinear processes is measured with a CCD camera. During a measurement the quality of the data should be checked. Therefore, I wrote a software program that controls the camera and visualizes the data of the camera.

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Introduction

A common method for detecting cancer inside the body is taking a biopsy [1]. A biopsy is a medical operation where a small piece of tissue is removed from a live person. The removed tissue is then placed under a microscope to look for cancer. This report is about a detection method which keeps the living tissue intact. The idea is that the microscope goes to the tissue, instead of that the tissue goes to the microscope. This research takes place in the Group Molecular Biophysics of the Debye Institute for Nanomaterials Sciences Utrecht University, and is performed in co-operation with the Erasmus MC Rotterdam.

Pulsed laser light is sent to the living tissue via a fiber. The laser induces two-photon excitation and second-harmonic generation in the tissue [2]. A part of the light created by these two processes goes back through the fiber. The returned light is sent to a CCD camera to detect this light. The CCD camera sends the data of the measured light to the computer. The data analysis is performed by software in LabView and ImageJ. The data is visualized by images in these programs.

The project has been running for almost four years and almost reaches its end. Therefore, mouse experiments are planned in the Erasmus MC Rotterdam. The skin on the back of the mice will be imaged. The growth of tumors will be followed during a couple of weeks.

At the moment the problem is that all the fibers are broken, so they are removed from the setup. However, we still want to measure the skin of the mice. Therefore, another system is implemented. This system is less flexible than a fiber, but good enough to reach the skin of a mouse's back.

During a measurement the data is analyzed quickly. This fast data analysis gives an indication about the quality of the data. If the quality is good enough, a deeper analysis method searches for the tissues infected by cancer.

This report is my bachelor thesis. My contribution lies in the control of the CCD camera and a fast data analysis to check its quality. To fulfill these two tasks, I wrote software programs in LabView.

1 Theory

Two nonlinear optical processes are important to image living tissues with a laser. These two processes are two-photon excitation and second-harmonic generation. Different natural fluorophores and collagen can be detected by the use of these processes. The information about changes in fluorophores and collagen gives an indication of the presence of tumors in the living tissues.

1.1 Nonlinear optical processes

When light passes through a material it can excite molecules from the ground state to an excited state. An example of such an excited state is a rotation between two parts of the molecule. Usually one photon excites the molecule to an excited state. This process is called one-photon excitation, see the left part of figure 1. After a short period of time, the molecule returns to his ground state. While the molecule is returning back to its ground state, one photon emits. This procedure is called fluorescence. Fluorescence has a typical lifetime of 10 ns [2].

During the process of fluorescence, there is a loss of energy. The photon that excites the molecule requires a higher energy than the energy between the ground and excited state. The difference between the two energies is called the Stokes shift. This means that the emitted photon always has a lower frequency than the incoming photon. The frequency of the emitted photon depends on the molecule. This can be used to distinguish between different kinds of molecules by detecting the emitted photons.

The intensity after the process one-photon excitation depends linearly on the intensity of the incident light beam. Such a process is called a linear optical process. Two examples of nonlinear optical processes are two-photon excitation and second-harmonic generation [4]. They are discussed in the following two paragraphs.

Two-photon excitation is almost the same as one-photon excitation. It is another way to excite a molecule. Two photons with not enough energy for one-photon excitation excite the molecule simultaneously. This is called two-photon excitation, see the right part of figure 1. The intensity after two-photon excitation increases as the square of the intensity of the incident light, therefore it is nonlinear.

A total different process is second-harmonic generation. Two photons merge together to one photon with twice the energy. This photon has half the wavelength and double the frequency of the first two photons. This process is a scattering process, generating an amount of light which is scattered backwards. Like two-photon generation, the intensity of the high energy photons increases as the square of the intensity of the incident light. The intensity of the high energy photons also depends on the material the light goes through.

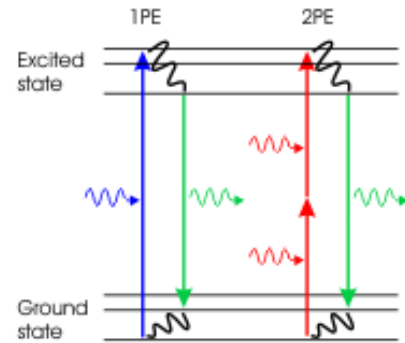


Figure 1: A Jablonski diagram to illustrate the difference between one-photon excitation (1PE) and two-photon excitation (2PE) [3]. In the left part a blue photon excites the molecule. After the excitation the Stokes shift follows. To finish the process, the molecule emits a green photon. In the right part two red photons excite the molecule, the rest of the process is the same as in the left part.

1.2 Nonlinear optical microscopy to detect tumors

Nonlinear optical microscopy is used to detect tumors. Nonlinear optical microscopy is based on nonlinear optical processes. A nonlinear optical process is a process in which the intensity of light after the process depends not only linear on the intensity before the process. The use of these processes in microscopy is called nonlinear optical microscopy. Two-photon excitation and second-harmonic generation are used in this microscopy experiment. The incoming light is emitted by a laser, which is focused on the spot you are interested in. For example on a mouse's back *in vivo*.

Different types of bio-molecules have different energy levels between the ground and excited states, so they emit different colors of light when the excited molecules fall back into their ground state. You can use the fluorescence of these molecules to detect and distinguish them. Two-photon excitation is used to excite the molecules in this experiment, because it has several advantages above one-photon excitation.

There are three advantages. The first two are due to the fact that two-photon excitation requires less energetic photons than one-photon excitation. This reduces the damaging effect of the photons, which is important for *in vivo* experiments. Secondly, the penetration range of the light is increased in the sample. The last advantage is the possibility to make 3D-images. This is a possibility, because two-photon excitation requires a high density of light. Fluorescence is only seen at the position where the beam is focused, not along the whole beam, see figure 2. You only detect the molecules in a small volume. Divide your sample in a 3D-raster of such volumes and measure each volume to create a 3D-images of your sample.

In conclusion, natural fluorophores of bio tissues can be imaged by two-photon excitation. The two natural fluorophores NADH and FAD are important in our case. They play a role in the energy metabolism in cells. Cancer affects the energy metabolism, thereby changing the ratio between NADH and FAD. This ratio can be measured with the use of two-photon excitation.

Furthermore, collagen is a material that stimulates second-harmonic generation. We add this effect to our fluorescence information to get better data, because collagen is also affected by cancer. Namely, cancer disturbs the structure of collagen. The non-symmetric electronic structure of collagen is the reason that it stimulates second-harmonic generation. Cancer breaks down the collagen matrix and induces a lower intensity of second-harmonic generation.

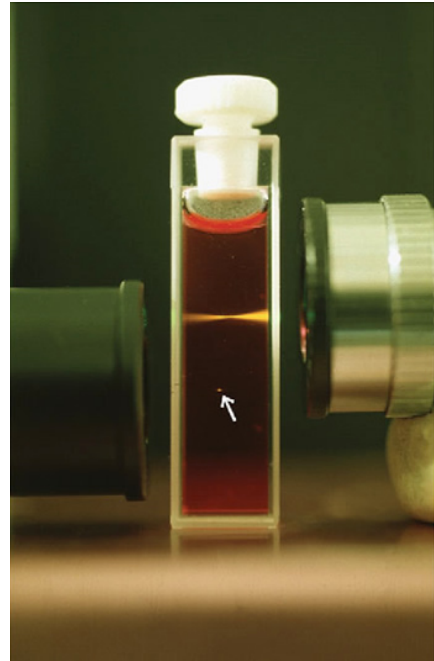


Figure 2: Fluorescence induced by one-photon excitation (above) and two-photon excitation (below) [5].

2 Setup

The setup is in the Erasmus MC Rotterdam, instead of our own Debye institute of the Utrecht University. It is build on a moveable table to be flexible in a hospital. A problem with the moveable table was that during a transport in the Erasmus MC the wheels under the table were broken off. The wheels could handle the weight, but not when they were exposed to side forces. The table was temporarily not mobile, but the wheels are repaired.

The table consists of two layers above each other. The lower layer is the support for the upper layer. The upper layer is an optical table where the measurement takes place. The upper layer consists of a lot of different parts. One of them is of great importance in this thesis, the CCD camera. Therefore, the camera is discussed in more detail. For further reading I recommend Anton de Boer's report (2013) [6].

The wheels are not the most important problem of the setup. The light should be transported by a fiber to the sample. The problem is that the fibers used in this experiment are very sensitive. All the fibers are damaged at the moment. There is a possibility to repair them, but this failed the last two times. To continue the project, another system is temporarily installed to do the mice measurements. This system is sufficiently well for measurements on the skin of a mouse, but for sure not enough to replace a biopsy inside a human body.

2.1 Total setup

The main components on the optical table are labeled in figure 3. They are all covered by one black plastic box. The box separates the optical setup from the outside world. This protects the instruments from dust and the light cannot escape to damage your eyes. The intensities of the beam starting from label 1 to 4 of figure 3 are even too high in this plastic box. The plastic will melt away if the beam hits the box. To prevent the box from melting, a black anodized aluminium box is placed around this part. The detector labeled with 9 is also surrounded by such a box, because of the sensitivity of the detector.

You need a light source to talk about two-photon excitation and second-harmonic generation. The light source is a Coherent Inc. Chameleon Ultra II Titanium Sapphire Laser (1 in figure 3). The wavelength of the light emitted by the laser is tunable between 680 and 1080 nm. The wavelength is 755 ± 5 nm in this experiment. The spread in the wavelengths is huge in comparison with a conventional laser. Usually, the spread is 1000 times smaller. This difference is caused by the fact that we use a pulsed laser. The intensity is above its half maximum for only 140 fs, this time is called the pulse width. The amount of pulses per second is 80 MHz.

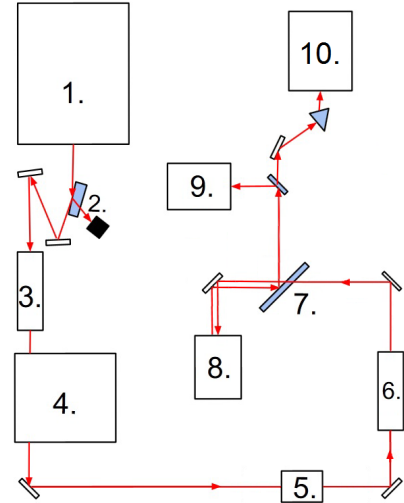


Figure 3: The setup illustrated in a schematic diagram [6]. The light emitted by the laser(1) follows the red arrows. The light passes some optical instruments(2,3,4,5,6) to optimize the laser bundle. The incoming and outgoing light from the sample holder(8) is separated by a dichroic mirror(7). Finally the light is sent to one of the detectors(9,10).

To avoid the huge spread in the wavelengths you can take a continuous beam, but there are two important advantages of a pulsed laser. The first advantage of a pulsed laser is that all the power is concentrated in a short period of time, instead of being spread over the whole period. So, a pulsed laser generates a higher peak intensity. A high peak intensity increases the change that photons are simultaneously at the same position. This is what you want, if you make use of two-photon excitation and second-harmonic generation. The other advantage lies in the performance of the laser. The laser is more off than on, so the heat has better chance to escape.

The averages output power of the laser is around 4,0 W. This power is far too high, it could damage some of the optical instruments in the setup. Therefore, a wedge made of glass is placed in front of the laser (2 in figure 3). The wedge reflects and refracts light. The reflected light goes further and the refracted light is dumped. The dumping of light induces a lower power, so the intensity decreases too.

Still, the intensity is too high, but we do not want to lose another huge amount of power. To lower the intensity even more, a beam expander is placed (3 in figure 3). A beam expander increases the diameter of the laser bundle, causing the intensity to decrease. A beam expander consists of two lenses. The first one converges the light, so the bundle is focused. After it is focused, the diameter grows. The second lens is placed at a point where the diameter is bigger then the original diameter. This lens converges the light back to a parallel bundle.

The intensity of the light is now low enough to pass through the rest of the optical instruments. Starting with the group velocity dispersion compensator (4 in figure 3). The group velocity dispersion compensator corrects the width of each pulse of the laser. The laser emits light with wavelengths of 755 ± 5 nm. Different wavelengths have another velocity when they travel through a medium. This velocity difference induces a broader pulse width, because some light falls behind of the rest of the light. This effect is called dispersion. The group velocity dispersion compensator gives the slower light the lead such that the pulse width is minimal at the sample. The minimal pulse width indicates a maximum intensity peak, which is what you want.

However, dispersion is not the same in each medium. For example it is minimal in air, but it plays a significant role when light travels through a fiber. At the moment we replaced the fiber by another setup. This means that the group velocity dispersion compensator is superfluous right now. It is not removed because it can compensate for the small dispersion of the rest of the optical parts, like lenses and filters. Again, it will play an important role when the fiber is back.

The laser travels from the group velocity dispersion compensator to the ND filters (5 in figure 3). ND filter stands for natural density filter [7]. This is a filter that reduces the intensity of light equally over all the wavelengths. Different filters are placed, so you can change the intensity manually. You want to use a power of less than 20 mW to examine the mouse, while still detecting fluorescence. Therefore, the ND filters are placed to be flexible with the intensity.

After these filters, a beam compressor is placed (6 in figure 3). This has an inverse effect as a beam expander. When we expand or compress the light, all the light gets focused somewhere in the middle. Pinholes are placed in these focus spots. In this way, the beam expander and compressor are both spatial mode filters.

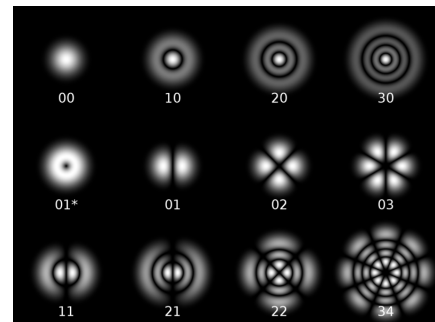


Figure 4: Some cylindrical transverse modes [8].

The spatial mode filters are necessary when we use the fiber. The fiber is a double clad photonic crystal fiber. The light can travel in both directions at the same time. The restriction is that the laser light must be in a single mode. Single mode means that the area transverse on the propagation of the light is in a single cylindrical mode, see figure 4. The pinhole of the spatial mode filters blocks all the fringes of the modes, so only the clean gaussian beam 00 remains. The pinhole in the compressor is removed at the moment, because we do not use the fiber.

The laser light is now ready to do a measurement. The light goes through a dichroic mirror (7 in figure 3) to separate the laser light and the light after it passes the sample holder (8 in figure 3). The sample holder is above all the optical instruments. So the light is pointed upwards to go through a scanner to reach the mouse on the sample holder. The scanner moves in zigzag pattern during a measurement, so we can make a 2D image.

The laser beam generates two-photon excitation and second-harmonic generation in the mouse. A part of the light goes back down. This light is reflected by the dichroic mirror, because the wavelength is decreased. This light is sent to a photomultiplier detector (9 in figure 3) or a CCD camera (10 in figure 3) by a flip mirror. The photomultiplier can only measure the intensity and is used to check the alignment of the light. The CCD camera is used to collect data during a measurement. The CCD camera is explained in detail in the next subsection.

2.2 CCD Camera

The camera which is used to collect data during a measurement is the Andor iXon 3 860 Electron Multiplier Charged Coupled Device (EM-CCD) [9][10]. The most important part of a CCD camera is the chip. The light that you want to measure is sent towards the chip. The chip converts the incoming photons into electron charges. To explain the possibilities of the camera I followed the processes from the incoming light beam to the data on the computer.

Before you start a measurement the temperature is set. The camera has a cooler that can reach a temperature between -120°C and 20°C . We use -70°C during measurements. The idea behind the cooling of the camera is to minimize the background signal of the chip. The background signal is the appearance of electron charges on the chip without the presence of photons. This background noise depends on the temperature. It decreases by cooling the chip.

After the cooling you can start a measurement. During a measurement light goes towards the camera. Just before the light arrives at the camera the light passes through a prism, see figure 5. Different colors of light have a slightly different refractive index in the prism. The different colors of the incident beam are separated in the outgoing light beam. The outgoing light is focused on the CCD camera. Now the location on the chip corresponds to the color of the light. The prism is placed, because the intensity of the incoming light is too low to use color filters.

After the light passes through the prism it enters the camera and is focused on the chip. The chip is divided into two parts. One part can be exposed to light and the second one is used to readout the data. First, the light induces electron charges on the first part, then the electron charges are transported to the second part by a potential. During the time to readout the data you can expose the first part of the chip again. Therefore, you can expose the camera with light while you are still reading out the data of the exposure before.

The chip is divided into a square pattern of pixels. We can use a part of all the pixels of the chip and merge pixels to one “big pixel” to make the camera even faster, see figure 6. The time to record a spectrum is reduced to 0.14 ms. This time is reached by focussing the beam on 20×128 pixels. These pixels are divided into “big pixels” of 20×1 pixels. So, we created 128 data channels. The beam is aligned so that the 128 channels correspond to different colors of light. To take into account the background light, there are another 128 channels above the first 128 channels. We created a 2×128 grid which measure the spectral data and the background of the spectral data.



Figure 5: The prism in front of the CCD camera. The prism splits up the different wavelengths of the incoming light and the camera detects the spectrum of that light.

The created electron charges on the first part of the chip are transported to the second part of the chip. The electron charges are readout at the lowest pixel line of the second part of the chip. The electrons in the lowest line are accelerated by a potential. After the acceleration they collide on a silicon wall. The high energetic electrons collide on the low energetic silicon electrons. This low energetic silicon electrons get accelerated too, and collide again, so you get a shower of electrons. The analogous electron signal is now intensified. The system that intensifies the analogous signal is called an electron multiplier. The intensified analogous electron signal is a current. This current is converted into a 14 bit number.

The potential in the electron multiplier can be set between 20V and 40V [11]. These two voltages correspond to a gain of 0 and 250 respectively. We used the gain 250. This gain is used to reach a pixel readout rate of 10MHz with an uncertainty less than one electron charge. If the rate is 10MHz and the electron multiplier is not used, than the readout uncertainty is 48 electron charges. Therefore, a electron multiplier is used to be accurate at high speeds. The accuracy and speed are important factors in this experiment. The accuracy is important because the intensity of fluorescence is low and the speed is important because the measurements are *in vivo*.

The digital data needs to be saved on the computer. To be faster, the camera has its own buffer. So there is not a direct connection between the measured data and the hard disc of the computer. The computer can only read data from this buffer. This is a circular buffer. If it is full, the new data is saved over the oldest data. To be sure you do not lose any data, you can readout the buffer during a measurement.

The CCD chip

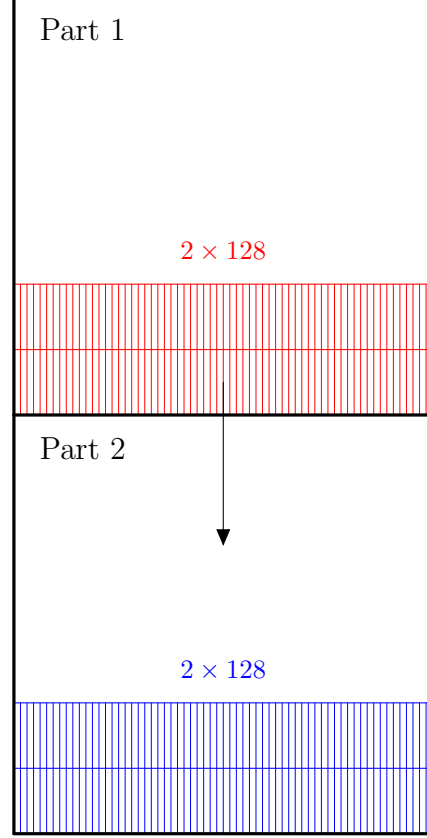


Figure 6: A schematic illustration of the two parts of the chip. Part 1 can be exposed to light. The red “big pixels” detect the light. Then a potential shifts the electrons from the red part to the blue part. The data is read from part 2.

3 Data acquisition

The CCD camera is controlled by the software LabView. LabView is a visual programming language [12]. It controls the camera and imports the raw data of the camera to the computer. The first data analysis is also performed in LabView. This analysis produces images which visualize the raw data. These images show the quality of the raw data. Furthermore, the data is saved by LabView. Then the software ImageJ can perform a more advanced data analysis.

A part of the sample is divided into a 256×256 grid. The camera records a spectrum at all the 65536 points of the grid. A point in the grid is called $p_{k,l}$, where k and l both run from 1 to 256.

3.1 LabView

The data of each single spectrum is represented by an one dimensional array in LabView. The array contains the information of three different things. The length of the array is equal to the amount of “big pixels” mentioned in subsection 2.2. The values in the array represent the intensity of light in one such a “big pixel”. The order of the numbers in the array corresponds to the position on the CCD camera.

LabView can receive the spectral data of the points separately or all at the same time. These data directly from the camera is called the raw data. I used the option to get the raw data of the spectra separately. Therefore, a quick analysis of the data during the measurement is possible. More about the method of receiving the spectra separately stands in subsection 4.2.

During a measurement there is just enough time to visualize the data in different kinds of images, they are discussed in the next subsection. The goal of this images is to get a first impression of the quality of the raw data. Furthermore, the raw data can be saved. It is saved in such a way that we can apply more detailed analyzing methods in ImageJ.

The data is saved in so called .spe files. The total length of the raw data is saved together with the raw data. The total length is the grid size times the amount of “big pixels”. Actually, this means that the total length is 256×65536 or 128×65536 if the background light is taken into account or not respectively, see subsection 2.2. The raw data is represented by a long row of numbers. The first 128 numbers are the spectral information of the first point of the grid. The next 128 are the spectral information of the background light of the first point or are the spectral information of the second point respectively. The long row of numbers of the .spe file is ordered in this way.

The focus of my thesis was to write a software program in LabView to control the camera, receive the data, save the data and to do a quick analysis to visualize the data.

3.1.1 CCD Camera

LabView contains functions to control the camera and to extract information from the camera. These functions are used so that the camera records spectra as required. First the camera gets the right settings. Then the camera is activated to record spectra. Each time when the camera records a new spectrum, the data of this spectrum is sent to the computer.

Examples of variables that must be set are the temperature, the size of the grid, the timing, the gain and the Iso Crop Mode. The timing is set by a connection with the scanner in the sample holder. The scanner ensures you that the camera records a spectrum in each point of the 256×256 grid. The gain is related to the potential that intensifies the analogous electron signals, see subsection 2.2. Then at last the Iso Crop Mode. This sets which pixel on the CCD chip is used and sets the size of the so called “big pixels”.

3.2 Images

Images are made to check the quality of the raw data, this is already mentioned in the subsection before. Two common ways are used to visualize the raw data in this thesis. These are the intensity image and the RGB Image. The phasor diagram is also used in addition to these two methods of visualization. The phasor diagram is a visualization of a spectrum.

An intensity image is a black-and-white image from 256×256 pixels in our case. It does not distinguish between different colors of light. The intensities of all the 128 color channels are added. The pixel of the lowest intensity of the 256×256 grid is black and the highest is white. The rest is a gray tint related with the intensity. This image throws away all the spectral information.

The RGB image is used to visualize the spectral information in a 256×256 image. The image is almost the same as the intensity image, but before all the color channels are added each channel is multiplied by the correspondent RGB values of the visible light spectrum. The color in one pixel in the RGB image is the average color of the correspondent measured spectrum.

A total different way to represent the spectral content of the raw data is the phasor diagram. The following procedure gives the simplest phasor diagram. The intensity of channel n is multiplied by $\exp(2\pi in/128)$, with n an integer between 1 and 128. Then you add all the elements in the complex plane. Note that this is the first component of the Fourier transform of the spectral information of one point of the grid. So we calculate for all the points $p_{k,l}$ in the 256×256 grid the following:

$$F_{k,l} = \sum_{n=1}^{128} I_{k,l,n} \exp\left(\frac{2\pi in}{128}\right) \quad \text{where } I_{k,l,n} \text{ is the normalized intensity of channel } n.$$

The normalized intensity $I_{k,l,n}$ is the intensity of the corresponding channel n divided by the total intensity at the point $p_{k,l}$.

The phasor is a square grid that divided the $[-1, 1] \times [-1, 1]$ square of the complex plane in discrete pieces, not necessarily a 256×256 grid. A empty phasor is represented by a square matrix with only zero elements. All the calculated Fourier transforms $F_{k,l}$ correspond to a matrix position. Each time a Fourier transform belongs to a particular matrix position, the corresponding matrix element is set one higher. The visualization of this matrix is the phasor diagram. The zero elements corresponds with black and the highest value in the phasor is blue. The rest is a color tint between black and blue.

Actually, the phasor diagram is the unit circle. The angle is related to the average of the intensity channels. For example, if the average is at channel n , then is the angle $2\pi in/128$. The other variable is the radius. The radius gives an indication about the width of the spectrum. This ensures that the background light which is constantly spread over the channels goes to the zero coordinate in the phasor. In conclusion, a point in the phasor diagram tells something about the shape of the corresponding spectrum.

4 The software

During my bachelor thesis I focused on the CCD camera of the setup discussed in subsection 2.2. The aim was to control the CCD camera with software written in LabView. During the writing of the software multiple goals were set about how the camera was to be controlled. The most of them, at least the important ones, has been achieved.

4.1 The goals of the software

The initial ideas on this project were to extract the raw data and to plot an image that shows the quality of the raw data. Later on the goals got more concrete and new goals were added. The goals were divided into main and secondary ones. They are summed up in the two lists below.

Main goals:

- Extract the raw data without the loss of raw data.
- Save the raw data.
- The speed of the scanner should be the restrictive factor, not the speed of the camera.
- Data processing should be faster than the camera can record spectra.
- At least an intensity or RGB image at end of a measurement.
- The calculations which are needed to create a image should be performed during the measurement.
- Control the temperature of the camera.

Secondary goals:

- User friendly, control the basics easily.
- User friendly, the possibility to control the advanced settings during a measurement.
- Live imaging.
- A plot of the phasor image.
- Use the phasor to unmix fluorophores for a first indication of the presence of cancer in the sample.

4.2 The process of writing the software

During the writing of the software there were some main problems to solve. Therefore, I had to make choices so that most of the goals were achieved. The important choices are described below. Furthermore, the software is tested during a measurement with real fluorescence. This was the final test of the software. The results of this test are also mentioned.

4.2.1 Problems and choices

I started with writing the software without knowing anything about other software programs from the Molecular Biophysics Group. Later on, it turned out that there were some relations. Two of them are saving the raw data and the RGB values of the visible spectrum. The raw data needs to be saved in the right way so that it fits into an analysis method in ImageJ. The other problem was the construction of the RGB image. I needed the RGB values of the visible spectrum. I used the values of *Mathematica*, but the Molecular Biophysics Group used their RGB values of the visible spectrum. This was solved easily, after I was informed about these things.

The goal to get the data analysis faster than the camera was the most restricting factor, but I made an analysis method that is fast enough to be faster than the time it costs to record a spectrum. Therefore, the data of one spectrum can be analyzed between the recording of this spectrum and the following one. In this case, I used the following structure to analyze the spectra. The computer checks whether there is a new spectrum. If not, he just asks again. If there is, the computer gives the command to readout the oldest spectrum, and then it is deleted from the buffer of the camera. After this, the analysis of the spectrum is fulfilled. This process is repeated for every spectrum. Even if the analysis lay behind for a short period of time, then it can catches up the camera. This structure also solves the problem with the size of the circular buffer of the camera, mentioned in subsection 2.2.

Before a measurement started the temperature of the camera is set. After one measurement the software stays running so that the temperature remains constant. Before you start a next measurement, you can change all the settings except the temperature. I divided the control panel in different tabs to split up the basics and the more advanced settings. Therefore, you can control the basics easily and you can still change the advanced settings. I also wrote a manual to make it more user friendly.

Another goal was live imaging, but this is a time consuming process. It appears that the creation of a live intensity image, live RGB image, or a live phasor diagram was possible. If the values needed for another image besides the one that was live are calculated a problem arises. The problem was that the updating time of a live image was too slow to keep up the speed of the camera. The solution was to plot the data of one row of the grid at once, instead of plotting each point separately. Now it was possible to calculate a intensity image, a RGB image and a phasor diagram while showing one of the three live during a measurement. You can choose which one of the three runs live; the other two are plotted after the measurement.

The final problem was to implement a method to unmix fluorophores. This goal arose at the moment that the structure of the software was almost finished. The idea was to use the calculations which are made to create the phasor diagram. Actually, this has not been done before in my Molecular Biophysics Group. So I looked for a way to implement it. My first results are discussed in section 5.

4.2.2 Results of the final test

During the writing of the software the setup was not ready to do fluorescence measurements. Therefore, the camera could not take images of fluorescence. The camera recorded only darkness. The darkness was enough to test the connection between the camera and my software in LabView. This darkness caused a problem. The time required to record a spectrum was unknown. The problem was solved by using a fast camera setting. This fast setting is necessary to do *in vivo* measurements. The time to record a spectrum was 0.14 ms.

Just before the end of my bachelor thesis the setup was able to do fluorescence measurements. Therefore, a final test is performed. During this test, images are taken from a piece of paper. Hereby, the actual time to record a spectrum is used. This time is 0.18 ms.

The difference between the estimated and the actual time was a consequence of the alignment of light on the camera chip. The height of the light was 40 pixels instead of the 20 pixels mentioned in subsection 2.2. The use of a bigger area of the chip requires a longer readout time.

Another new part of the final test was the camera in combination with the scanner. The scanner is also controlled by software in LabView. During the test we combined both softwares in LabView. The conclusion is that LabView is able to run both simultaneously. Spectra were recorded while the scanner changes the position of the focus in the sample. Only the camera and the scanner were not yet synchronized.

From this final test also follows that the time factor is really important. Before the settings were changed to do a real measurement the software crashes. For some kind of reason the software was become faster while the camera runs at the same speed as before. Time delays are added to solve this problem in the software. This time delays are not yet optimized. The final test was not long enough to optimize the time delays.

4.3 The final software

I wrote a software program to control the CCD camera and to do a fast data analysis to check the quality of the data. All the goals mentioned in subsection 4.1 are fulfilled, except for the last one.

First, the settings of the camera can be set in the software. Then the software is ready to start a measurement. During a measurement all the spectra of the 256×256 grid are sent one by one from the camera to the computer. Each spectrum is analyzed before the next spectrum is sent to the computer. After the analysis of one line of the grid, an image is updated to create a live image. This image is an intensity image or a RGB image or a phasor diagram. When all the lines are analyzed the software creates an intensity image and a RGB image and a phasor diagram. During the measurement the raw data is also automatical stored in an array. Before the next measurement is started the array can be manually saved in a .spe file. The biggest disadvantage of the software is that the data cannot be analyzed again in this software, but only in ImageJ.

I also wrote a manual so that anyone can use the software easily. The manual is attached in appendix 1. The manual shows screenshots of the panels which are required to control the camera. To get a better idea of the software program, I also attached a screenshot of the underlying code in appendix 2. The code on this screenshot brings in the raw data of the camera at Get Oldest Image. Then, the data is analyzed to create a live phasor diagram. The intensity image and RGB image are created at the end.

5 From the phasor to biological interpretation

The last goal mentioned in subsection 4.1 is the use of the phasor to unmix fluorophores for a first indication of the presence of cancer in the sample. This goal appears at the end of my research. The setup is build to find tumors in a living tissue, so it would be nice if a first indication of cancer can be implemented in the quick analysis.

5.1 Unmix fluorophores by a phasor

Each spectrum belongs to a point in the phasor. This is already mentioned in subsection 3.2. Furthermore each fluorophore has a characteristic spectrum. Therefore, a fluorophore is always located at the same position in the phasor.

Important fluorophores are NADH and FAD in our measurement, see subsection 1.2. The Group Molecular Biophysics has already measured the spectra of NADH and FAD. If the 128 channels correspond to wavelengths from 350 nm to 700 nm then the corresponding phasor points are $-0.58 + i0.57$ and $-0.82 - i0.03$ respectively. The phasor distinguishes NADH and FAD.

If you add two points in a phasor you get a point in the middle of those two points. So if you add two spectra with the same total intensity, then the phasor point goes also to the middle. If the intensities are not equal, then the point stays still on the line between the original points, but not anymore in the middle. The point is shifted to the original point which belongs to the spectrum with the highest intensity. The addition of two points in the phasor is a weighted average relative to the total intensities.

If two different fluorophores in a sample are measured simultaneously, the spectra are added. If you know the theoretical phasor points of the two fluorophores in the sample, then you can determine the ratio between the two fluorophores. The phasor point of this measurement is the weighted average between the two theoretical phasor points. The ratio of the distances in the phasor between a theoretical point and the measured point is the same ratio as the ratio between the fluorophores.

The case of two different fluorophores in a sample is one specific situation. The following possibility is three fluorophores in a sample. The measured phasor point is the weighted centroid of the triangle between the three theoretical phasor points. If we know the three corresponding theoretical phasor points, the ratio between the three fluorophores can still be determined [13].

If we go further to four or more fluorophores, the phasor is insufficient to unmix fluorophores. This follows from the fact that the first order Fourier transform is not injective. This means that different spectra belong to the same phasor point, so you cannot go from a phasor to the spectral information. The phasor contains less information than the spectral information.

5.2 Multiple measurements

Methods to determine the ratio between different fluorophores from one measurement is discussed in the previous subsection. In our case we take 65536 spectra instead of one. The ratio of the fluorophores in each single phasor point can be determined as before, and the total intensity of each point is measured. From this the ratio of the fluorophores in the whole grid of 65536 points can also be calculated. This method is a generalization of determining the ratio from only one measurement.

Instead of a generalization, you can look for better methods. Some points are maybe only background signal. These points are centered around zero in the phasor. Therefore, a method which excludes the phasor points around zero is better. Such a method is to look only at regions of interest. A region of interest is an area around interesting fluorophores, like NADH and FAD. The ratio of the phasor points in this area are taken into account to calculate the total grid ratio.

To select the right region of interests, the possibility to repeat the analysis will be better. Mentioned before in subsection 4.3, this is not possible with the structure that is used. Therefore, I think that a method to unmix fluorophores fits better in the precise data analysis after a measurement. The disadvantage of this method of unmix fluorophores is that it cannot run live.

6 Conclusion

I joined the Molecular Biophysics Group of the Utrecht University to write a software program. One of their researches is that they will use nonlinear optical microscopy imaging to detect tumors *in vivo*. A high pulse laser induces two-photon excitation and second-harmonic generation in a sample. A CCD camera detects the light emitted by these two processes. The initial intention was to write a software program to control the CCD camera and to write a quick analysis method to check the raw data. During the writing process of the software precise goals were set, see subsection 4.1. All the goals are fulfilled, except the last one.

The last goal was to use the phasor to unmix fluorophores for a first indication of the presence of cancer in the sample. I tried to implement this goal. The conclusion from section 5 is that this fits better in the precise data analysis after a measurement, because sometimes you need multiple tries to find the best method to unmix the fluorophores.

The final test mentioned in subsection 4.2.2 shows that the software is ready to combine it with the software of the scanner. It also shows that the time factor between the recording speed of the camera and the running speed of the data analysis in the software is a sensitive factor. This time factor causes sometimes a software crash, so this should be improved.

7 Further research

The software of the camera is tested only once in real action. To optimize the software there are more tests necessary. The test discussed in subsection 4.2.2 shows the sensitivity of the time factor between the camera and the data analysis. Secondly, the software of the scanner should be combined better with the software of the camera. In such a way that these two run not only simultaneously, but that they are also synchronized.

A first indication of the quality of the data is implemented in the software. You cannot conclude something about the presence of cancer from this indication. The first ideas to implement a method to conclude the presence of cancer are discussed in section 5. To add such a method, further research is necessary. Even if this method is found, it fits possibly better in the deeper analysis.

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Appendix 1: Manual of software Control_CCD_Camera

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Introduction

This is a manual about the software located at C:\Data\David\Control_CCD_Camera. By using this software, you can control the CCD camera of the setup. Furthermore, the raw data of the camera is visualized and can be saved.

1 Main functions

Start the program at the same way as all LabView programs by clicking on the arrow button. First the CCD camera turns on, see the indicator CCD ON. Then the cooler turns on. You can see when the cooling is done at the Temperature tab. It is done if Temp Reached and Temp Stable are both bright light green or you switched the Do Not Wait button to ON.

From this moment you can start a measurement at one of the tabs Intensity, RGB Image or Phasor. If a measurement is successful, the indicator Measurement turns bright light green. Before each measurement you can change some settings at the Settings tab. The three tabs Intensity, RGB Image or Phasor are almost the same, the main difference is the live imaging. The name of the tab indicates which image is live during a measurement. After each measurement it is possible to save the raw data at the Save Data tab. To stop the program, you should hit the STOP button. The cooler will slowly return to 0°C. When this temperature is reached, the cooler turns off, then the camera and this program will shut down. The slow return to 0°C is really important, if it goes too fast, the camera can be damaged. So if this program crashes, it should be running again so fast as possible to set the cooler to on.

The Errors tab can help you to find problems with the camera, if the camera does not respond as usual.

Each tab is discussed individually in the next five sections. All the indicators and controls are described in tables. If there is a default value mentioned, it is a control, if not it is an indicator.

2 Settings

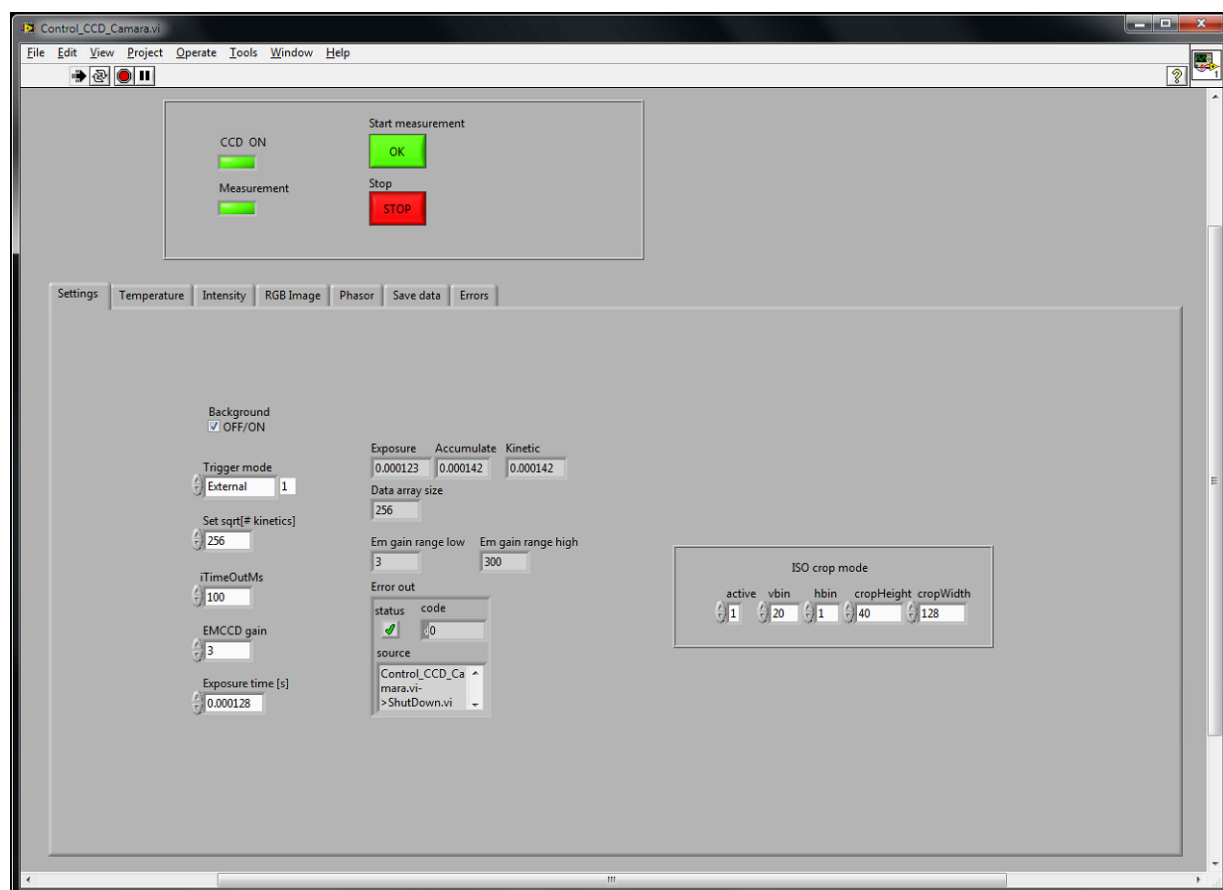


Figure 1: Front panel switched to Settings tab

Name	Default	Discription
Background correction	ON	During each measurement you also detect the background noise on the chip to correct your data.
Trigger mode	External 1	The mode that activates the camera. External 1 is related to the scanner.
Set sqrt[# kinetics]	256	The edge length of the square grid.
iTimeOutMs	100	After the start of the acquisition the program waits for the first spectral data. This is the maximal time in milliseconds the program will wait.
EMCCD gain	3	The gain of the camera.
Exposure time[s]	0.000128	This sets the exposure time in seconds, if trigger mode is Internal 0.
Exposure		The real exposure time in seconds.
Accumulate		This indicator is not relevant in this thesis.
Kinetic		The time in seconds that the camera needs to detect one pixel.
Data array size		This should be equal to Set sqrt[# kinetics]. This is implemented to check the amount of spectra the camera will take during one measurement.
EMCCD gain range low		Minimum EMMCD gain that is possible.
EMCCD gain range high		Maximum EMMCD gain that is possible.
Error out		When you stop the program, this gives the status of the program.

Table 1: Settings

The ISO crop mode is an option that is also used. You force the camera to regard only at a part of the chip instead of the whole chip. The CropHeight and the CropWidth sets the amount of chip pixels that you want to use. And the Vbin and Hbin give the size of the chip that forms one camera pixel. If the background correction button is turned to ON, the CropHeigth is doubled. For example, if you turn the Background correction to ON and you use the default settings of the ISO crop mode, then the total of camera pixels becomes 256. For the default settings, see table 2.

Name	Default	Discription
Active	1	Turns the ISO crop mode on(1) or off(0).
Vbin	20	Sets the vertical bin of the ISO crop mode.
Hbin	1	Sets the horizontal bin of the ISO crop mode.
CropHeight	20	Sets crop height.
CropWidth	128	Sets crop width.

Table 2: ISO crop mode

3 Temperature

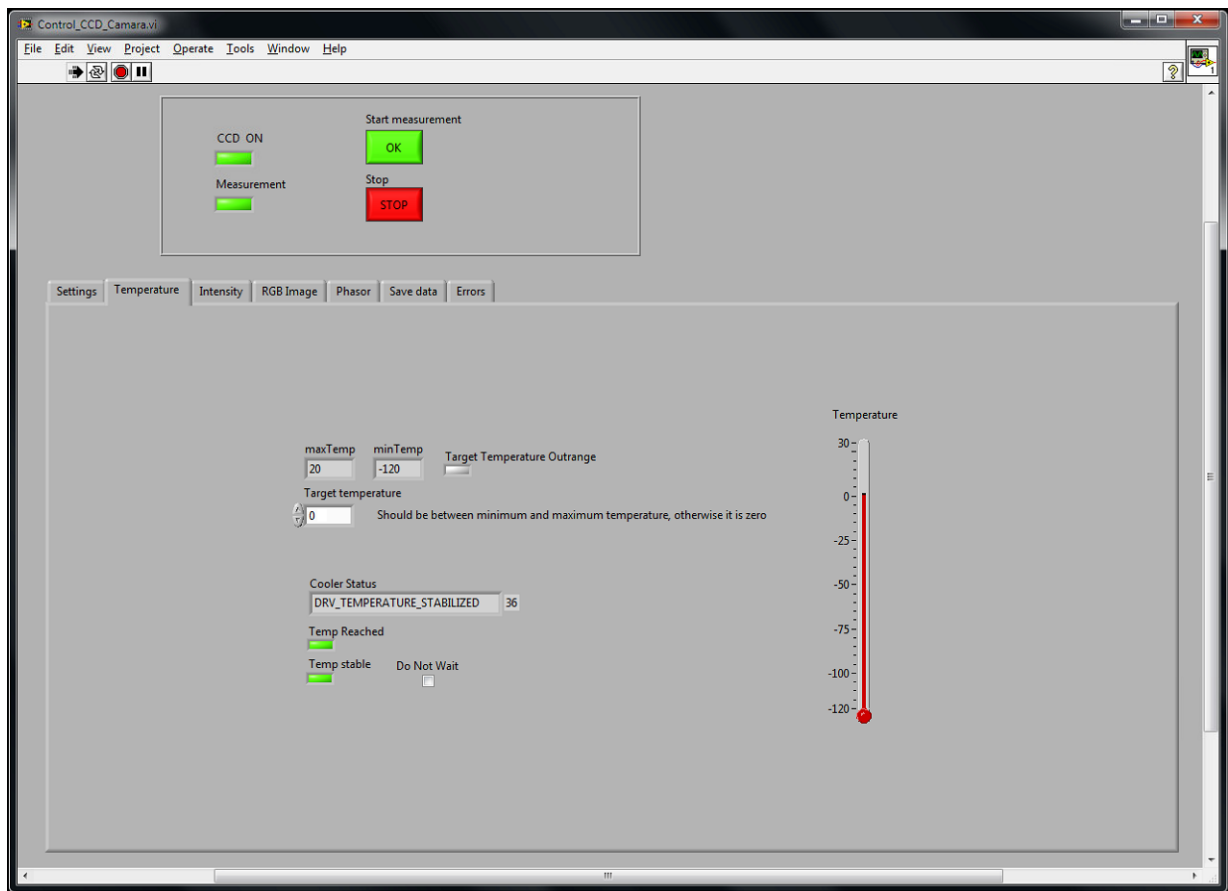


Figure 2: Front panel switched to Temperature tab

Name	Default	Discription
Temperature		Shows the temperature in degree Celsius during cooling and heating.
MaxTemp		The maximum temperature the cooler can reach.
MinTemp		The minimum temperature the cooler can reach.
Target temperature	-70	Sets the cooler temperature. This can only be set before the software runs.
Target temperature outrange		If Target temperature is not between MaxTemp and MinTemp, it turns red. Then the cooler is set to 0°C.
Cooler status		Returns the status of the cooler.
Temp reached		When the Target temperature is reached within 3°C it turns bright light green.
Temp stable		When the Target temperature is stable and in 1° from the Target temperature it turns bright light green.
Do Not Wait	OFF	You can skip the waiting time of the cooling by turning this button to ON (Not recommended).

Table 3: Temperature

4 Intensity, RGB Image and Phasor

The three tabs Intensity, RGB Image and Phasor look all the same. The difference between this tabs is which image returns live images during a measurement. For example the Intensity tab, see figure 3 and table 4. These three tabs are the only tabs where you can start an measurement.

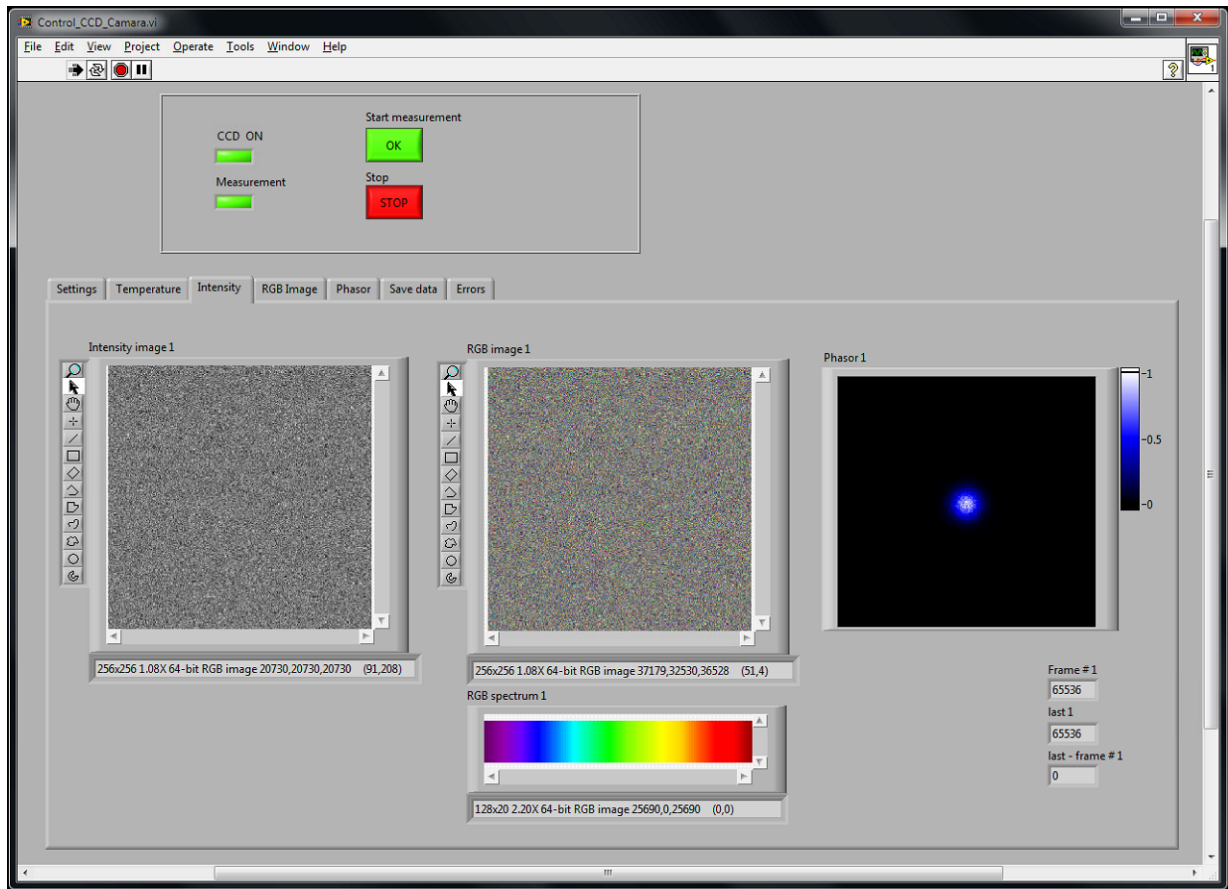


Figure 3: Front panel switched to Intensity tab

Name	Discription
Intensity image 1	Gives an intensity image of the last measurement that is recorded at the Intensity tab.
RGB image 1	Gives a RGB image of the last measurement that is recorded at the RFB Image tab.
Phasor 1	Gives a Phasor image of the last measurement that is recorded at the Phasor tab.
RGB spectrum 1	Shows the color scheme of the 128 color channels to create the RGB image.
Frame # 1	The picture number that gets evaluated.
Last 1	The amount of pictures that the camera has recorded.
Last - Frame # 1	Gives the difference between Last 1 and Frame # 1, this indicates the status of the measurement.

Table 4: Intensity

5 Save Data

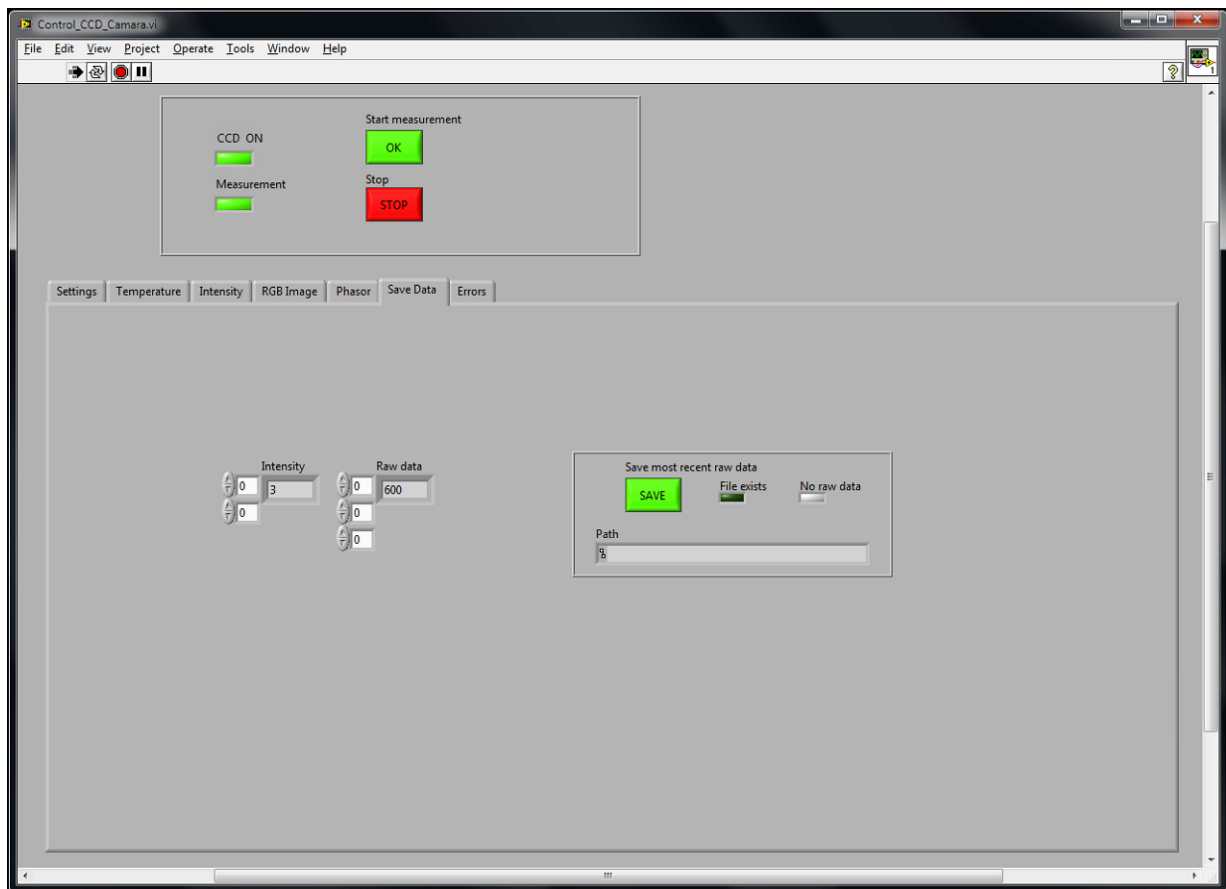


Figure 4: Front panel switched to Save Data tab

Name	Discription
Intensity	Array of intensity values of the last measurement.
Raw data	Array of raw data of the last measurement.
Save most recent raw data	Save Raw data.
Path	Path of the last saving.
File exists	Turns to red if Path already exists before saving.
No raw data	Turns to red if there has not been a measurement.

Table 5: Save Data

6 Errors

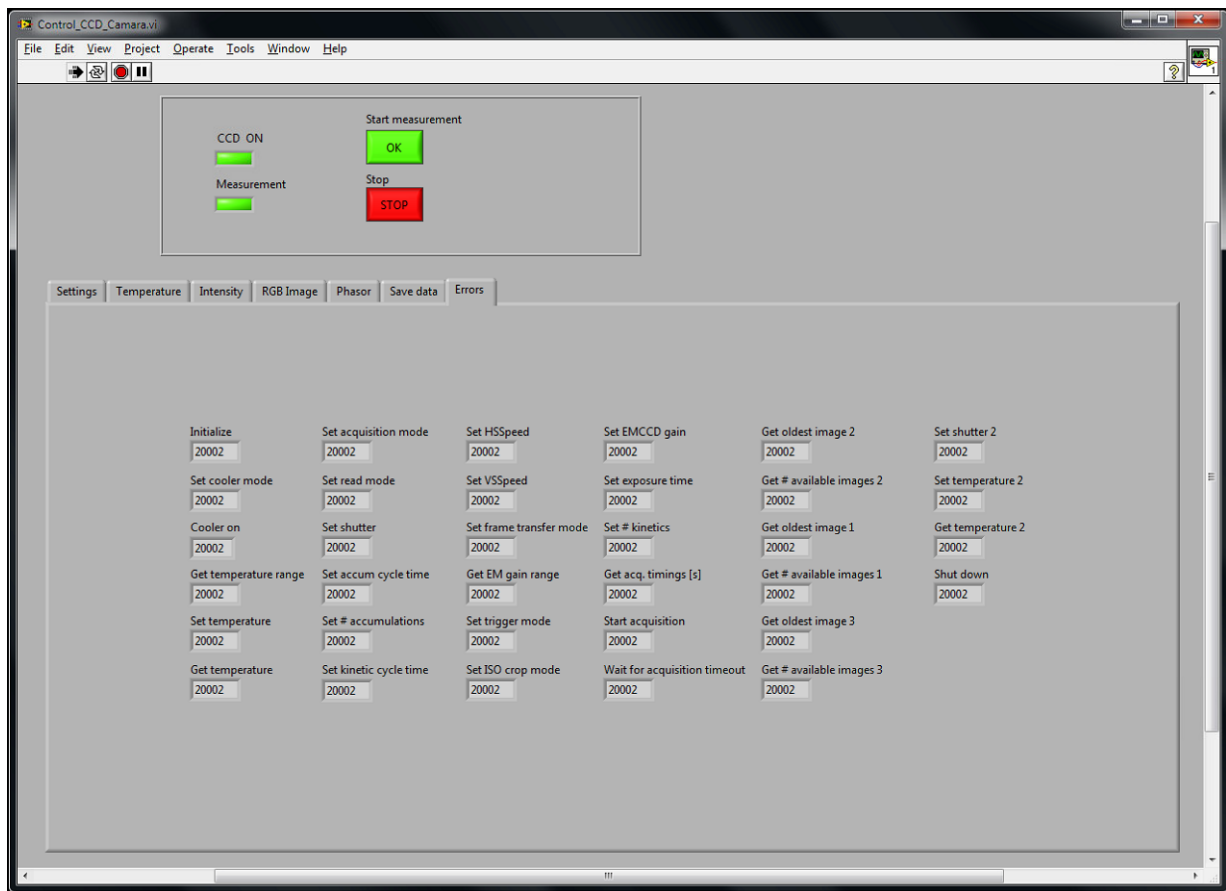


Figure 5: Front panel switched to Errors tab

This tab shows the status of all the camera functions. The error indicators are placed in the same order as in the block diagram. The number 20002 means no error. All the error codes are shortly described in the appendix: USER'S GUIDE TO: ANDOR™ TECHNOLOGY SDK page 291. For more detailed information I refer to whole document USER'S GUIDE TO: ANDOR™ TECHNOLOGY SDK.

References

USER'S GUIDE TO: ANDOR™ TECHNOLOGY SDK

SECTION 12 - ERROR CODES

CODE	ERROR	CODE	ERROR
DRV_ERROR_CODES	20001	DRV_P1INVALID	20066
DRV_SUCCESS	20002	DRV_P2INVALID	20067
DRV_VXDNOTINSTALLED	20003	DRV_P3INVALID	20068
DRV_ERROR_SCAN	20004	DRV_P4INVALID	20069
DRV_ERROR_CHECK_SUM	20005	DRV_INIERROR	20070
DRV_ERROR_FILELOAD	20006	DRV_COFERROR	20071
DRV_UNKNOWN_FUNCTION	20007	DRV_ACQUIRING	20072
DRV_ERROR_VXD_INIT	20008	DRV_IDLE	20073
DRV_ERROR_ADDRESS	20009	DRV_TEMPCYCLE	20074
DRV_ERROR_PAGELOCK	20010	DRV_NOT_INITIALIZED	20075
DRV_ERROR_PAGE_UNLOCK	20011	DRV_P5INVALID	20076
DRV_ERROR_BOARDTEST	20012	DRV_P6INVALID	20077
DRV_ERROR_ACK	20013	DRV_INVALID_MODE	20078
DRV_ERROR_UP_FIFO	20014	DRV_INVALID_FILTER	20079
DRV_ERROR_PATTERN	20015	DRV_I2CERRORS	20080
DRV_ACQUISITION_ERRORS	20017	DRV_DRV_I2CDEVNOTFOUND	20081
DRV_ACQ_BUFFER	20018	DRV_I2CTIMEOUT	20082
DRV_ACQ_DOWNFIFO_FULL	20019	DRV_P7INVALID	20083
DRV_PROC_UNKNOWN_INSTRUCTION	20020	DRV_USBERROR	20089
DRV_ILLEGAL_OP_CODE	20021	DRV_IOCERROR	20090
DRV_KINETIC_TIME_NOT_MET	20022	DRV_NOT_SUPPORTED	20091
DRV_KINETIC_TIME_NOT_MET	20022	DRV_USB_INTERRUPT_ENDPOINT_ERROR	20093
DRV_ACCUM_TIME_NOT_MET	20023	DRV_RANDOM_TRACK_ERROR	20094
DRV_NO_NEW_DATA	20024	DRV_INVALID_TRIGGER_MODE	20095
DRV_SPOOLERROR	20026	DRV_LOAD_FIRMWARE_ERROR	20096
DRV_SPOOLSETUPERROR	20027	DRV_DIVIDE_BY_ZERO_ERROR	20097
DRV_TEMPERATURE_CODES	20033	DRV_INVALID_RINGEXPOSURES	20098
DRV_TEMPERATURE_OFF	20034	DRV_BINNING_ERROR	20099
DRV_TEMP_NOT_STABILIZED	20035	DRV_ERROR_NOCAMERA	20990
DRV_TEMPERATURE_STABILIZED	20036	DRV_NOT_SUPPORTED	20991
DRV_TEMPERATURE_NOT_REACHED	20037	DRV_NOT_AVAILABLE	20992
DRV_TEMPERATURE_OUT_RANGE	20038	DRV_ERROR_MAP	20115
DRV_TEMPERATURE_NOT_SUPPORTED	20039	DRV_ERROR_UNMAP	20116
DRV_TEMPERATURE_DRIFT	20040	DRV_ERROR_MDL	20117
DRV_GENERAL_ERRORS	20049	DRV_ERROR_UNMDL	20118
DRV_INVALID_AUX	20050	DRV_ERROR_BUFFSIZE	20119
DRV_COF_NOTLOADED	20051	DRV_ERROR_NOHANDLE	20121
DRV_FPGAPROG	20052	DRV_GATING_NOT_AVAILABLE	20130
DRV_FLEXERROR	20053	DRV_FPGA_VOLTAGE_ERROR	20131
DRV_GPIBERROR	20054	DRV_BINNING_ERROR	20099
DRV_DATATYPE	20064	DRV_INVALID_AMPLIFIER	20100
DRV_DRIVER_ERRORS	20065		

