H E F A

Helium Fluorescentie & Absorptie

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1. SETUP

The HEFA apparatus offers the ability to measure absorbance, various kinds of fluorescence, linear dichroism and circular dichroism spectra. Measurements can be done at room temperature as well as cryogenic temperatures. Figure 1.1 gives an overview of the HEFA apparatus.

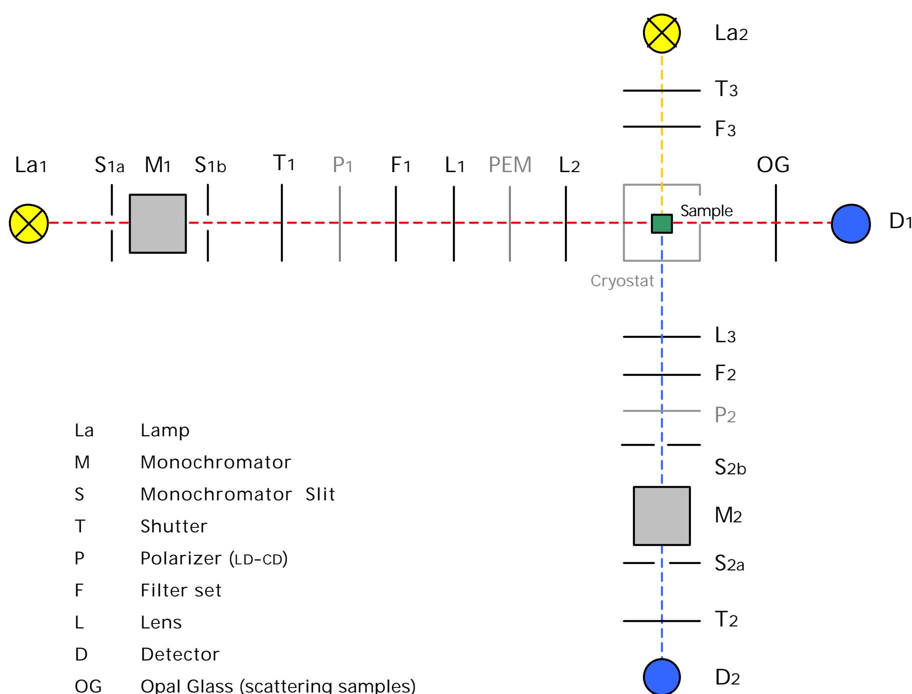


Figure 1.1 HEFA setup

The following table summarizes the various types of experiments in relation to the components of HEFA.

Measurement	Type	Lamp	M1	M2	D	PEM	Lock-in
Absorbance	A	La1	scans		D1		
Linear Dichroism (LD)	A	La1	scans		D1 (PM)	V	2F
Circular Dichroism (CD)	A	La1	scans		D1 (PM)	V/2	F
Fluorescence emission	F	La1	fixed	scans	D2		
Fluorescence excitation	F	La1	scans	fixed	D2		
Fluorescence polarization	F	La1	scans	fixed	D2		

2. MEASUREMENTS

The following paragraphs describe the setups and measurement procedures to use for the various measurements. Appendix A. gives a summary of the theory of measurement-methods.

2.1 Absorbance

Absorbance spectra are measured with the following setup.

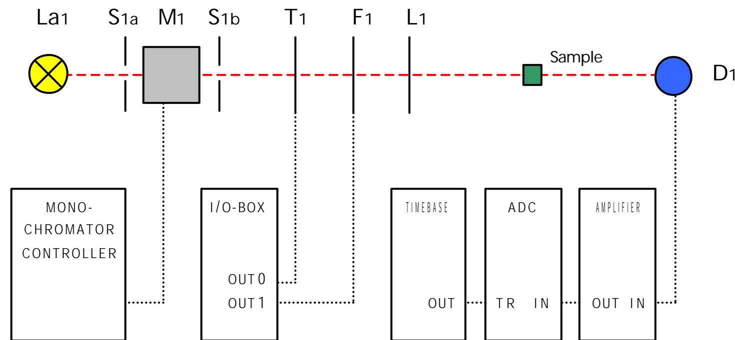


Figure 2.1 HEFA absorbance setup

Absorbance, transmittance and absorptance are defined as follows.

$$A = {}^{10}\log \frac{I_0}{I}$$ absorbance, optical density

$$T = \frac{I}{I_0}$$ tranmittance

$$1 - T = \frac{I_0 - I}{I_0}$$ absorptance

Intensity I_0 is measured with a blank sample, intensity I with the sample of interest.

The method used to measure intensity with the photodiode, leads to a signal even in the complete dark situation. This *dark-signal* has to be taken into account in the measurement.

The spectra are measured in two parts: a red part and a blue part. At a certain wavelength in the scan, $l_{crossover}$, filters are changed. This is why we speak of a filter *set*.

Below, a simplified version of the measurement procedure is shown.

insert blank sample

determine monochromator slit widths (and gain and filter settings) with blank sample

measure(I_0)

insert sample of interest

measure(*I*)

Where *measure*(I_m) is as follows:

```
adc.setLength( L)
timebase.setFrequency( F)
filter.setCrossoverWavelength( ?crossover)

for scan = 1 to N do

    filter.set( ?begin)
    monochromator.set( ?begin)

    shutter.close()
    dark-level-begin = 100 x adc.mean() / adc.fullscale()
    shutter.open()

    for ? = ?begin to ?end step ?step do
        filter.set( ?)
        monochromator.set( ?)
        Is[?] = 100 x adc.mean() / adc.fullscale() - dark-level-begin
    done

    shutter.close()
    dark-level-end = 100 x adc.mean() / adc.fullscale()

    Im += Is
    A = log( I0/ I)

done
```

Primary parameters	Recorded information
N • number of scans	date, start time, end time
l$?_{begin}$ • begin wavelength [nm]	sample name,
l$?_{end}$ • end wavelength [nm]	sample OD
l$?_{step}$ • wavelength step [nm]	temperature [K]
	cuvet form, thickness, material
Secondary parameters	slit widths M1, M2 [mm]
G • amplifier gain Z • amplifier filter [Hz]	ADC gain (1)
F • ADC sampling rate [Hz]	*dark-level-begin*
L • samples for *adc.mean*()	*dark-level-end*
l$?_{crossover}$ • filter crossover wavelength [650nm]	**l**?- I_0 - *I*, *(A)*

2.2 Linear dichroism

2.3 Circular dichroism

2.4 Fluorescence emission

2.5 Fluorescence excitation

2.6 Fluorescence polarization

3. ANALYSIS AND DESIGN

The HEFA setup enables measuring absorbance spectrum, linear and circular dichroism spectra and fluorescence emission, excitation and polarization spectra.

The system should enable users

R1. to perform the various absorbance and fluorescence measurements effectively and efficiently.
R2. to save the measurement results and their context to data files.
R3. to view previously made measurements.

3.1 Users and usability requirements

End-users Two classes of users can be distinguished: HEFA user and Other staff.

HEFA user – those who actually perform measurements with the HEFA setup. The users are postdocs, Phds and students in physics with various levels of scientific experimental experience. All are direct users and for most of them – in the order of ten – HEFA is not their primary setup and they only will use it *intermittently*. There may be a couple of persons that will be very *experienced* with the HEFA setup. All users are experienced computer and MS-Windows users.

Other staff – a class of users that only wants to review previously measured spectra.

Usability requirements The system should enable both the experienced and the less experienced user to perform the measurements.

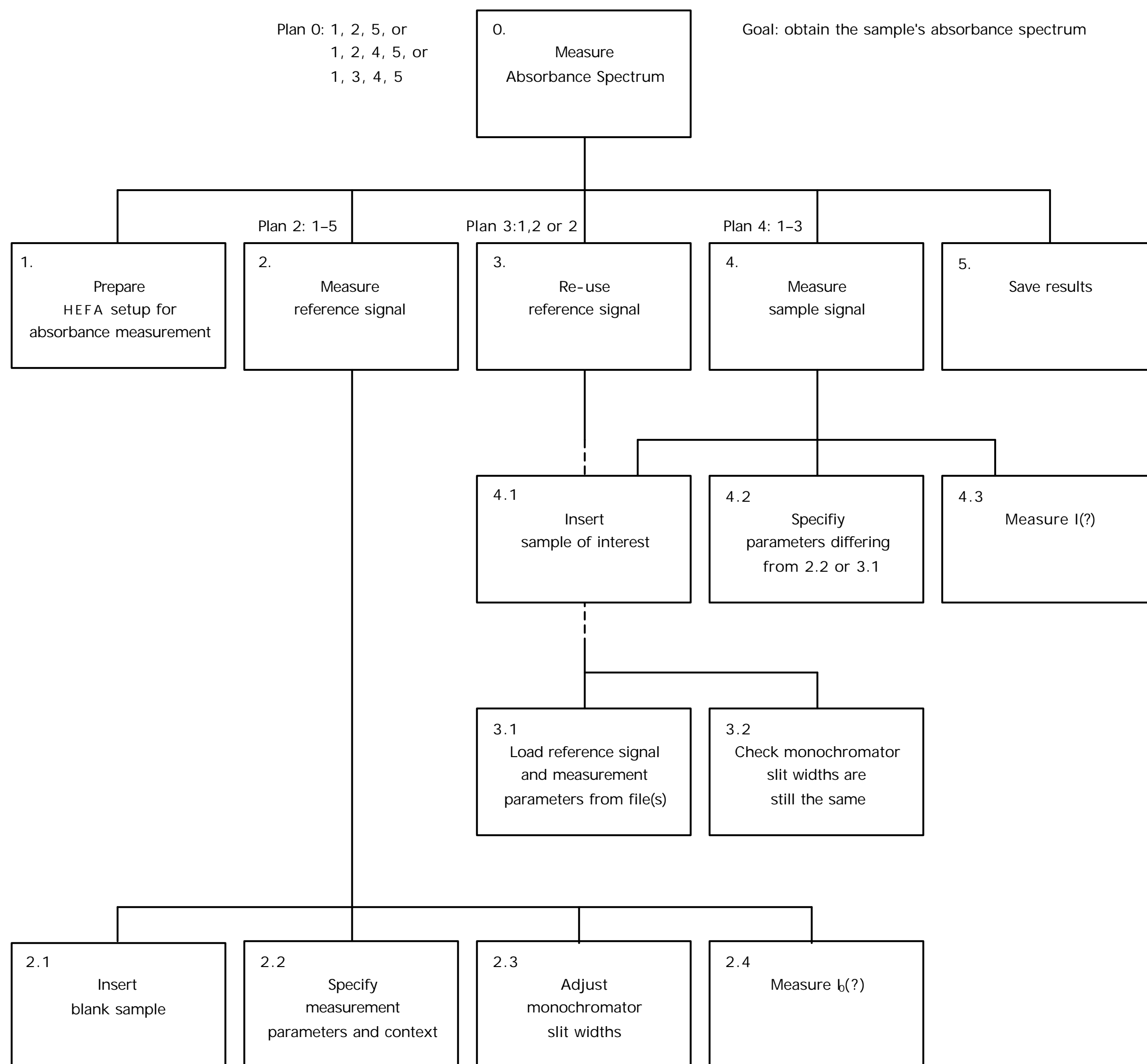
UR1. Low error rate. The system will guide users through the measurement, visualizing the (partial) results and providing other feedback on what is happening (R1).
UR2. Low error rate. The system will prevent the user to perform invalid actions (R1).
UR3. High efficiency. The system will automate as much as possible and provide remote monitoring. However, also opportunities to enable the very experienced people to do 'unanticipated' things will be looked for (R1).
UR4. High efficiency. The system will support measuring and saving the reference signal separately from the signal with the sample of interest and reloading this signal from a data file (R1).
UR5. High efficiency. The system will support saving and loading the program configuration (R1).
UR6. Interoperability. Measurement results and their context will be saved to data files in a format that can be easily used with other programs to enable further processing (R2).
UR7. User satisfaction. User will be 'in control'. Improve on existing system.

Evaluation criteria The usability requirements will not be measured.
Level to achieve The usability requirements will not be measured.

3.2 User tasks

Tasks The user tasks reflect the measurements as can be done with the HEFA setup. Another task is viewing previously measured spectra. Figure 3.1 on the next page shows the task model for measure absorbance spectrum.

Task scenarios For now, no scenarios will be made.



Tasks are performed at the HEFA setup, partly in the dark.
One reference measurement may be made for several measurements of the sample of interest. Note however that the setup must be untouched between these measurements, specifically, the monochromator slit widths may not be changed in between.

Figure 3.1 Task model for absorbance measurement.

3.3 User objects

Multiple models

UOM1 HEFA user – direct users that perform measurements.
UOM2 Other staff – 'viewers'.

User Objects

Source	User Object	Attributes
Users	Measurement	number of scans wavelength range (from, to, step)
	Context (measurement information)	date time from time to sample sample OD sample temperature monochromator slit widhts
	Configuration	ADC number of samples ADC sampling rate, filter set crossover wavelength filter set peak transmittance wavelength data directory
	Reference signal Sample signal Absorbance signal Data file Context file	
Task models	—	—
Data model	—	—

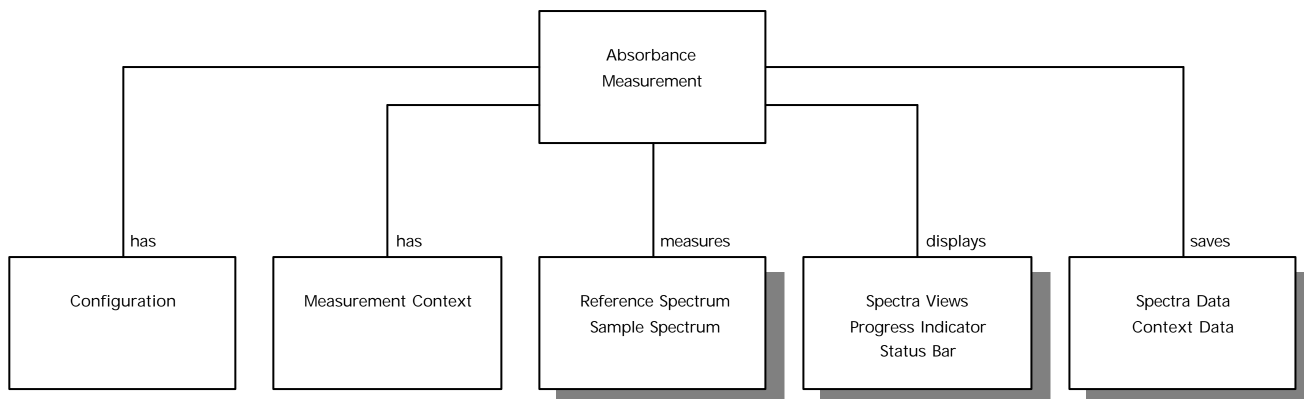


Figure 3.2 User object model for absorbance measurement.

Absorbance Measurement attributes

- Number of scans
- Spectrum begin wavelength
- Spectrum end wavelength
- Spectrum wavelength stepsize

Aborbance Measurement actions

Load Purpose is to load a reference signal into the buffer and skip measuring one.

Input — filename.

Effects — this loads a reference signal into the reference buffer and displays the spectrum. Further it makes changing the wavelength range impossible. A measurement now will consist of only the scans for the sample of interest.

Output — reference spectrum loaded and displayed.

Unload Purpose is to unload the reference signal from the buffer.

Input — none.

Effects — this removes the reference signal from the reference buffer and the spectrum view. It enables changing the wavelength range again. A measurement now will consist of a light beam intensity adjustment, a reference measurement and a measurement of the sample of interest.

Output — reference spectrum loaded and displayed.

Run Purpose is to start a measurement.

Input — the attributes.

Effects — depending on the measurement procedure, this performs a light beam intensity adjustment procedure, and a blank sample reference measurement, or loading of a reference spectrum, and the measurement of the intensity spectrum of the sample of interest and the construction of the absorbance spectrum. Spectra are also displayed.

Output — spectra collected and displayed.

Continue Purpose is to make extra measurement scans, either after a Stop, or when the number of scans has been incremented after completing the previously specified number of scans.

Rest as within Run.

Save Purpose is to save the measurement results.

Input — spectra and measurement context.

Effects — save data and context to files in the datadirectory. Automatically generate names like 001231xy.000 for the data and 001231tx.000 for the context files.
The naming scheme is {year}{month}{day}{code}.{sequence}.
Ask for confirmation to save the data to these files and allow for changing the names and directory.

Output — data and context files.

Stop Purpose is to complete current scan, then stop or continue the current measurement.

Input — none

Effects — complete the current scan, further behave as if the measurement is completed, so the sprectra can be saved.

Output — none

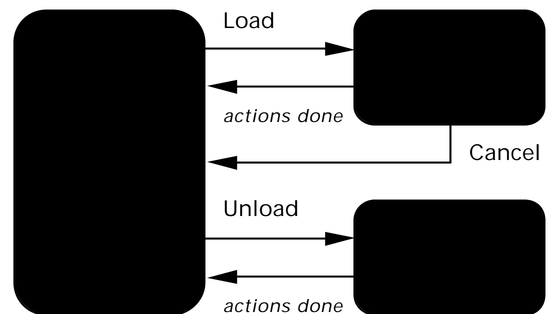
Stop now Purpose is to terminate measurement immediately without completing current scan.

Input — none

Effects —interrupt the current scan and discard it, further behave as if the measurement is completed, so the sprectra can be saved.

Output — none

Action	State-dependency
Load	only if idle
Unload	only if idle
Run	only if idle
Continue	only if Waiting,
Save	only if measurement completed
Stop	only if Scanning or Waiting
Stop now	only if Scanning or Waiting



State	Action	Next state	Outputs
Idle	Load	Idle	disable wavelength inputs
	Unload	Idle	enable wavelength inputs
	Run, ref. unloaded	Inserting blank	light measuring reference LED
	Run, ref. loaded	Inserting sample	light measuring sample LED
Inserting blank	Cancel	Idle	clear all LEDs
	Done	Adjusting	—
Adjusting blank	Cancel	Idle	clear all LEDs
	Done	Scanning blank	—
Scanning blank	*all scans completed*	Waiting blank	Run ? Continue
	Stop now	Waiting blank	Run ? Continue
	Stop	Stopping blank	*discard partial scan*
Stopping blank	*this scan completed*	Waiting blank	Run ? Continue
Waiting blank	Continue	Scanning blank	—
	Stop, Stop now	Inserting sample	light measuring sample LED
Inserting sample	Cancel	Saving	light ready LED, Continue ? Save
	Done	Scanning sample	—
Scanning sample	*all scans completed*	Waiting sample	Run ? Continue
	Stop now	Waiting sample	Run ? Continue
	Stop	Stopping sample	*discard partial scan*
Stopping sample	*this scan completed*	Waiting sample	change Run ? Continue
Waiting sample	Continue	Scanning sample	—
	Stop, Stop now	Saving	light ready LED, Continue ? Save
Saving	Cancel	Idle	?? Run
	OK	Idle	?? Run

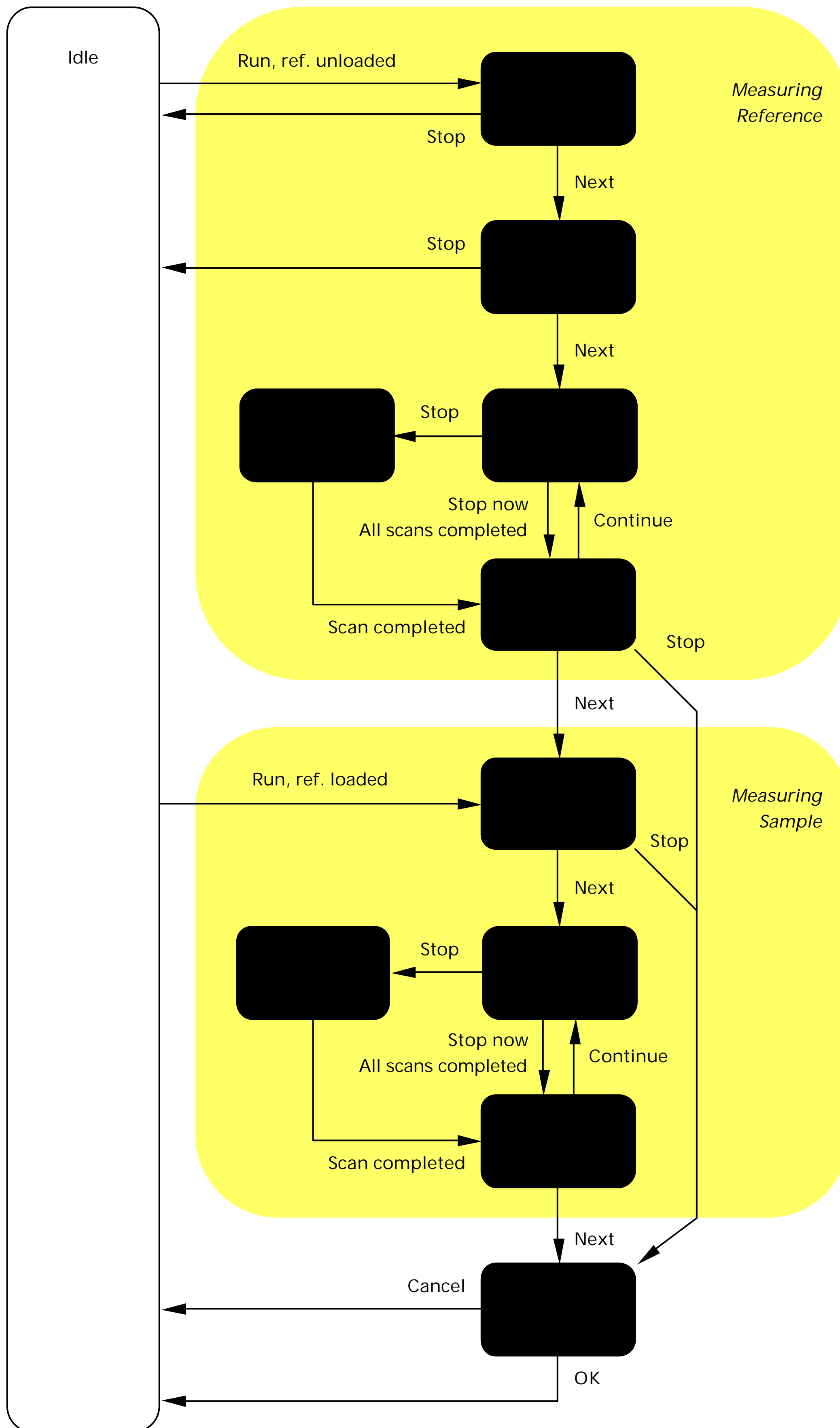


Figure 3.3 Measurement statechart.

Abbreviations

ADC	analog to digital converter
CD	circular dichroism
DAC	digital to analog converter
LD	linear dichroism
OD	optical density
PEM	photo elastic modulator

Glossary of user terms

Absorbance	measurement method based on effect that sample absorbs energy of light passing it, exciting pigment molecules, defined as: $^{10}\log \text{Intensity}_{reference}(\boldsymbol{I}) / \text{Intensity}_{sample}(\boldsymbol{I})$.
Amplifier	RULBUS programmable amplifier, lock-in amplifier.
Analog to digital converter	RULBUS module to convert analog voltage to a computer readable number.
Blank sample	cuvet with buffer or solvent only to measure reference signal.
Circular Dichroism	absorbance type measurement method using modulated polarized light.
Configuration	measurement parameters and information and program settings.
Context	information concerning the measurement, like temperature etc.
Crossover wavelength	wavelength at which the red and blue filters are changed; attribute of filter set.
Cryostat	device to cool sample to a very low temperature.
Data directory	directory to save files with measurement results and its context.
Detector	the light detector used: photo-diode or photo-multiplier for LD/CD.
Dichroism	linear, circular.
Filter	optical device to pass part of the spectrum of a light beam.
Filter set	assembly with two filters, one of which can be selected. e.g. for the red and blue parts of a spectrum.
Fluorescence	effect that sample emits light when relaxing from excited to ground state.
Fluorescence emission	fluorescence type of measurement method.
Fluorescence excitation	fluorescence type of measurement method.
Fluorescence polarization	fluorescence type of measurement method.
I/O controller box	RULBUS module for digital in- and output (SIFU).
Lamp	light source to illuminate the sample with monochromatic or actinic light.
Lens	optical device to focus light beam or make it parallel.
Linear Dichroism	absorbance type measurement method using modulated polarized light.
Lock-in amplifier	amplifier with synchronic detection of modulated signal.
Monochromator	optical device to produce a monochromatic light beam. See also Slit.

Glossary of user terms

Monochromator controller	RULBUS module to control a Bausch & Lomb monochromator remotely.
Optical density	optical attribute of a sample, defined as xxxxx.
Photo-elastic modulator	electronic/optical device to control polarization of a light beam.
Polarizer	optical device to influence polarization of a light beam.
Programmable amplifier	RULBUS module with programmable amplification factor and low pass filter.
Pump	pump with control unit to produce vacuum for ????.
Reference	intensity of a blank sample measured over a wavelength range.
Sample	cuvet with organic sample of which absorbance etc. is to be determined.
Samples	number of samples the ADC reads each wavelength point (attribute of ADC).
Sampling rate	number of samples the ADC reads each second (attribute of ADC).
Scans	number of signal measurements to average over.
Shutter	mechanical device to block or pass a light beam.
Slit	opening in monochromator's light beam of which the width can be adjusted mechanically. A monochromator has an input and an output slit.
Spectrum	intensity, absorbance etc. signal over a wavelength range.
Peak transmission wavelength	wavelength at which the filter set has the highest transmission; attribute of filter set.
Timebase	RULBUS module to generate timing pulses for the ADC trigger input.
Transmittance	effect that sample absorbs energy from light passing it, defined as: $\text{Intensity}_{sample}(I)$ / $\text{Intensity}_{reference}(I)$.
Wavelength	wavelength of the light leaving the monochromator, or of the light emitted by the sample.

3.4 Style guide

The style guide is only touched here very briefly.

GUI environment LabVIEW under MS-Windows.

Expected style Users will expect a Virtual Instrument front-panel presentation.

Window look Instrument-like front-panel with TABS to access various parts.

Standard menu items No menus will be used.

Window controls TABS, entry and display fields, drop-down lists, buttons.

Standard pattern of interaction (to be filled.)

Help system LabVIEW help system with tip strips and descriptions. All window controls.will provide appropriate help information.

3.5 Design GUI

- Supports user tasks.
- Presents the user's objects clearly.
- Conforms to the style guide.
- Meets usability requirements.

- What views of objects are required for tasks?
- How should these views be allocated to windows?
- What layout should be used for windows?
- How does the user navigate from one window to another?
- What menu items and other controls are required, and how do they behave?

- Window designs, including specification of interactive behaviour.
- Window navigation design.

3.6 Prototype GUI

- How can the user perform the task scenarios using the GUI?
- Are extra views of objects required?
- Should the windows be restructured to support tasks better?
- Is the inter-window navigation adequate to support tasks?
- How can 'what the person does' be simplified and streamlined?
- What problems does the user encounter?
- What improvements does the user suggest?

- A working prototype GUI
- Revisions to the GUI design

3.7 Evaluate GUI

- How usable is the GUI by the end-users, in terms of the usability criteria previously specified?
- What usability problems do users encounter?
- Does the GUI provide adequate support to all types of users performing their full range of tasks?

- An evaluation of the usability of the GUI design and prototype.
- Usability problems.
- Proposed revisions to the GUI design.

4. BIBLIOGRAPHY

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Photosynthesis

Photosynthesis is the process in which plants and some types of bacteria transfer the energy of (sun)light into chemical energy used for maintaining and the reproduction of the organism. Photosynthetic organisms can be divided into two groups according to the type of photosynthetic reaction applied by these organisms, the first group being the photosynthetic bacteria and the second group consists of algeae and higher plants. The latter group produces oxygen as a side-product of the photosynthetic reaction.

Independent of the type of photosynthetic reaction used, the photosynthetic system can always be divided into two parts, firstly the antenna and secondly the reaction center. In the antenna light is gathered through absorption by pigments, placing them in an excited state. These excitations are then transferred to the reaction center in which the energy is used to make a charge separation which can subsequently induce chemical reactions.

Light can be absorbed in the cell by different pigments, but all organisms contain at least one chlorophyll-like pigment and in most species carotenoids are present as well. Algeae and higher plants contain chlorophyll a (Chl *a*), and some species chlorophyll b as well. In bacteria more different types of bacteriochlorophyll (BChl) are found, BChl *a* through *g*. In most bacteria more than one type of pigment is present.

Theory of measuring-methods

In this report the photosynthetic system is looked at from a physics viewpoint, using physical experiments to obtain information about the system. The first process that occurs is of course the absorption of light by the pigments in the antenna. The pigment molecule comes in an electronic excited state and the energy can then either be transferred to another molecule or several other processes can occur. In this report we will confine ourselves to the transfer of energy to other molecules and only one of those other processes, fluorescence. An (overly) simplified energy scheme is presented in Figure A.1.

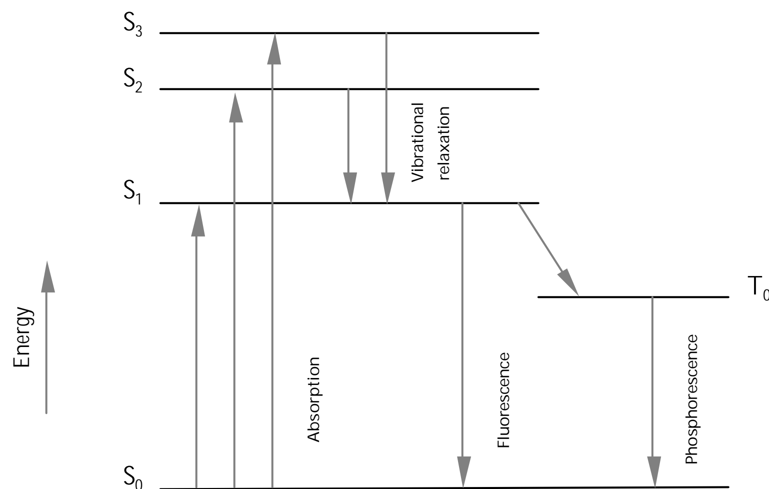

Figure A.1 Energy scheme of a pigment molecule

Absorbance

Normally a molecule is in the state with the lowest energy, the ground state called S_0. Absorption of a photon excites the molecule to a higher energy level. Depending on the energy, and therefor on the wavelength ($E = hc/\lambda$), of the absorbed photon the pigment is excited from S_0 into one of the higher energy states S_1, S_2, S_3 etc. Measuring the absorption of light while scanning through a wavelength interval produces an absorption spectrum. The absorbance A is proportional to the specific extinction ε of the sample at the wavelength at which absorption is measured. Two other properties the absorbance is proportional to are the concentration *c* of the sample and the optical pathlength *l* of light in the sample. For a parallel beam of light this dependence is expressed in v.Beer's law

$$A = {}^{10}\log \frac{I_0}{I} = \varepsilon \cdot c \cdot l$$

in which I_0 and I are the intensities of light going in respectively coming out of the sample.

Because of interactions of the pigment with it's environment and because of thermal effects the spectrum will not be made up of sharp absorption lines but of gaussian shaped bands. The band formed by exciting a molecule to the S_1 state is called the Q_y band. Excitation to S_2 gives rise to the Q_x band, and excitation to S_3 produces the Soret band, which is actually composed of two bands, B_x and B_y. Figure 2 shows the general shape of an absorption spectrum of the BChl *a* protein of the green sulfur bacterium *Prostecochloris aestuarii*.

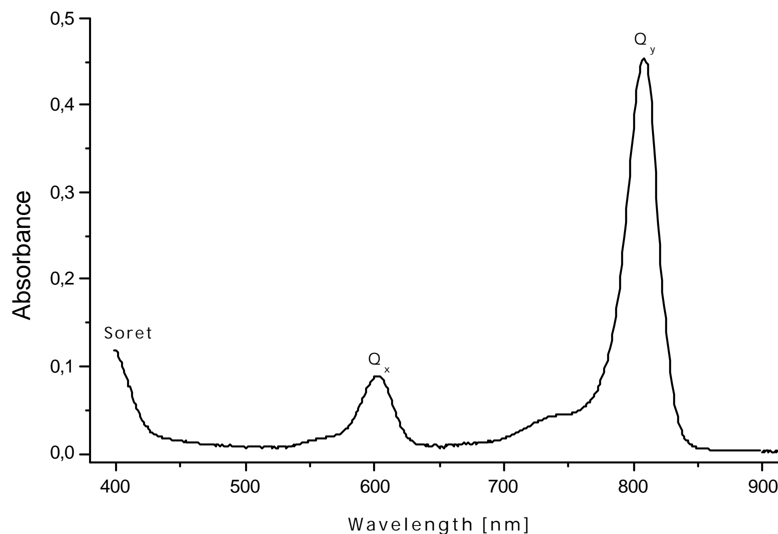

Figure A.2 Absorption spectrum of the BChl a protein from Prostecochloris aestuarii at room temperature

Each different type of molecule has different energy levels and therefor a different absorption spectrum. As a result the absorption spectrum can be used to get a general idea of the molecular contents of a sample.

Fluorescence emission

Once a molecule is in an excited state it will not remain there indefinitely but will relax to a state of lower energy until the ground state is reached. Relaxation from S_3 and S_2 to S_1 takes place by vibrational relaxation. In the S_1 state a molecule can either emit a photon, which is called fluorescence, or it can relax to the ground state of the triplet, T_0. Emission of a photon from T_0, called phosphorescence, can then occur. A fluorescence emission spectrum is obtained by measuring the emission of fluorescence at varying wavelength while exciting the sample at one wavelength (e.g. in the Q_x band).

Emitting aphoton represents a loss of energy to the photosynthetic process. A pigment can also relax to the ground state by transferring the energy to another molecule. It is this process which enables the photosynthetic system to transfer energy from the antenna to the reaction center. The efficiency of the photosynthetic process is directly dependent on the efficiency of this energy transfer.

Fluorescence excitation

Once the fluorescence emission spectrum is known, the intensity of light emitted at the maximum (or one of the maxima) of the fluorescence emission spectrum can be measured while varying the excitation wavelength. This produces a fluorescence excitation spectrum. From this spectrum the efficiency of energy transfer from states of higher energy to the fluorescing state (S_1) of a pigment, or, more important, from another pigment to the fluorescing pigment can be derived.

The fluorescence excitation is not proportional to the absorbance A, which is presented in the absorption spectrum but to the absorptance 1–T, since fluorescence takes place on a linear scale. The 1–T spectrum can be calculated from the absorption spectrum by using the formula

$$T = 10^{-A}$$

Both spectra are normalized at the peak corresponding to the emitting state. The fluorescence excitation spectrum will be identical to the absorption spectrum if the efficiency of energy transfer from the absorbing state to the emitting state is 100%. For lower efficiency the amplitude in the excitation spectrum is correspondingly lower. Relative amplitudes of peaks in the spectra can then be used to calculate the efficiency of energy transfer.

Fluorescence polarization

The two basic measuring methods, absorption and fluorescence excitation, can be elaborated on by using polarized light. For fluorescence excitation this results in fluorescence polarization spectra. A sample is excited by plane-polarized light and emission of plane polarized light parallel and perpendicular to the exciting light is then measured. The polarization p can be defined as

$$p = \frac{I_{||} - I_{\perp}}{I_{||} + I_{\perp}}$$

where $I_{||}$ and $I_{\perp}$ stand for the intensities of the emitted light polarized parallel respectively perpendicular to the exciting light for a 'random', isotropic system of unmoving, isolated molecules the following equation holds

$$p = \frac{3\cos^2 \boldsymbol{a} - 1}{\cos^2 \boldsymbol{a} + 3}$$

where $\boldsymbol{a}$ is the angle between the transition moments of the absorption- and the fluorescence-dipole. This way relative angles of the dipoles formed by molecules and their electrons can be ca1culated.

LD/CD

In absorption measurements one can use either plane polarized or circular polarized light. The difference in absorption of horizonta1ly and vertica1ly plane polarized light is ca1led linear dichroism (LD) and the difference in absorption of left and right circular polarized light is ca1led circular dichroism (CD).

LD measurements require a sample with an orientation superimposed on it. This is usually done by placing the sample in a gel with a rigid structure of polymers. The particles of the sample are held within this structure. Pressing the gel in one or two directions (uni- or biaxia1 pressing) will stretch this structure in the other direction(s) imposing a orientation on the particles of the sample. One can then define the linear dichroic rate (LDR or anisotropy) as

$$LDR = \frac{A_{\parallel} - A_{\perp}}{A} = \frac{3}{2}(1 - 3\cos^2 \phi)$$

in which A is the absorbance of the sample and ϕ the angle between the transition dipole and the normal to the plane of the membrane. $A_{\parallel}$ and $A_{\perp}$ are the absorbances of plane polarized light parallel or perpendicular to the superimposed orientation. When the orientation of the sample by pressing is not perfect, information derived from an LD spectrum will be qualitative and not quantitative. A positive signal indicates a small angle between the transition dipole and the plane of the membrane, a negative signal indicating a larger angle.

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